BIOSYNTHETIC STUDIES ON PSEUDOMONIC ACID

by

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A thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1989

For My Parents

Patience is a virtue...

DECLARATION

I declare that this thesis is my own composition, the work of which has been carried out by myself unless otherwise stated, and that it has not been submitted towards a higher degree or qualification. The research was undertaken in the Chemistry Departments of the Universities of Edinburgh (2 years) and Leicester (1 year), under the supervision of Professor T J Simpson, after the 1st October 1986, the date of my admission as a postgraduate student.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor T J Simpson, for all his helpful advice and encouragement. I would also like to thank my industrial supervisor, Mr S W Elson, for his help with the microbiological work. My thanks are extended to the technical staff within the Chemistry Departments of both Edinburgh and Leicester Universities. Finally, a grant from the SERC and financial support from Beecham Pharmaceuticals are gratefully acknowledged.

ABBREVIATIONS

CSA	Chiral solvating agent
DMSO	Dimethyl sulphoxide
DPPA	Diphenyl phosphorazidate
HMDS	1,1,1,3,3,3-Hexamethyldisilazine
HMG	β-Hydroxy-β-methyl glutarate
$LiAlH_4$	Lithium Aluminium Hydride
LDA	Lithium di-isopropylamine
MVA	Mevalonic acid
NAC	N-acetylcysteamine
PCC	Pyridinium chlorochromate
PDC	Pyridinium dichromate
PTSA	Toluene-4-sulphonic acid
THF	Tetrahydrofuran
TFEA	R-(-)-Trifluoro-anthryl ethanol

ABSTRACT

Chapter one reviews the literature published on the pseudomonic acids. The chemistry and structural characteristics of these compounds is covered together with the semi-synthetic modifications which have been investigated to enhance their anti-bacterial properties. Some of the synthetic routes to pseudomonic acid C are reviewed along with its subsequent conversion to pseudomonic acid A. The previous biosynthetic studies are described in detail.

Chapter two briefly reviews the use and methods of detection of radioactive and stable isotopes in biosynthetic studies. The maintance of the *P. fluorescens* culture and the conditions for the production and isolation of the metabolites are described. Intial incorporation experiments using [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{2}H_3$]acetate and $^{18}O_2$ gas are discussed and the results from these experiments allow for mechanisms for the formation of the pyran ring and unsaturated ester linkage to be proposed. Synthesis and incorporation of ^{14}C and $^{13}C_2$ labelled forms of β-hydroxy-β-methyl glutarate and its non-intact incorporation into pseudomonic acid is discussed. The synthesis of ^{13}C labelled malonate and the results of its incorporation into pseudomonic acid are described and indicate that malonate is an important precursor. Differential levels of enrichment were observed with malonate going preferentially into the 9-hydroxynonanoic

acid side chain.

In chapter three, a stepwise mechanism for the formation of pseudomonic acid is proposed and discussed. The synthesis of three of the proposed chain assembly intermediates, *ie.* acetoacetate; (2S,3S)-3-hydroxy-2-methylbutanoic acid and 5-hydroxy-4-methylhex-2-enoic acid are described. Results of intial incorporation studies using 5% $^{2}H_{2}O$ in the culture medium and [2-$^{2}H_{3}$]acetate are detailed together with the attempted incorporation of (2S,3S)-[2'-$^{2}H_{3}$]-3-hydroxy-2-methylbutanoic acid chain assembly intermediate.

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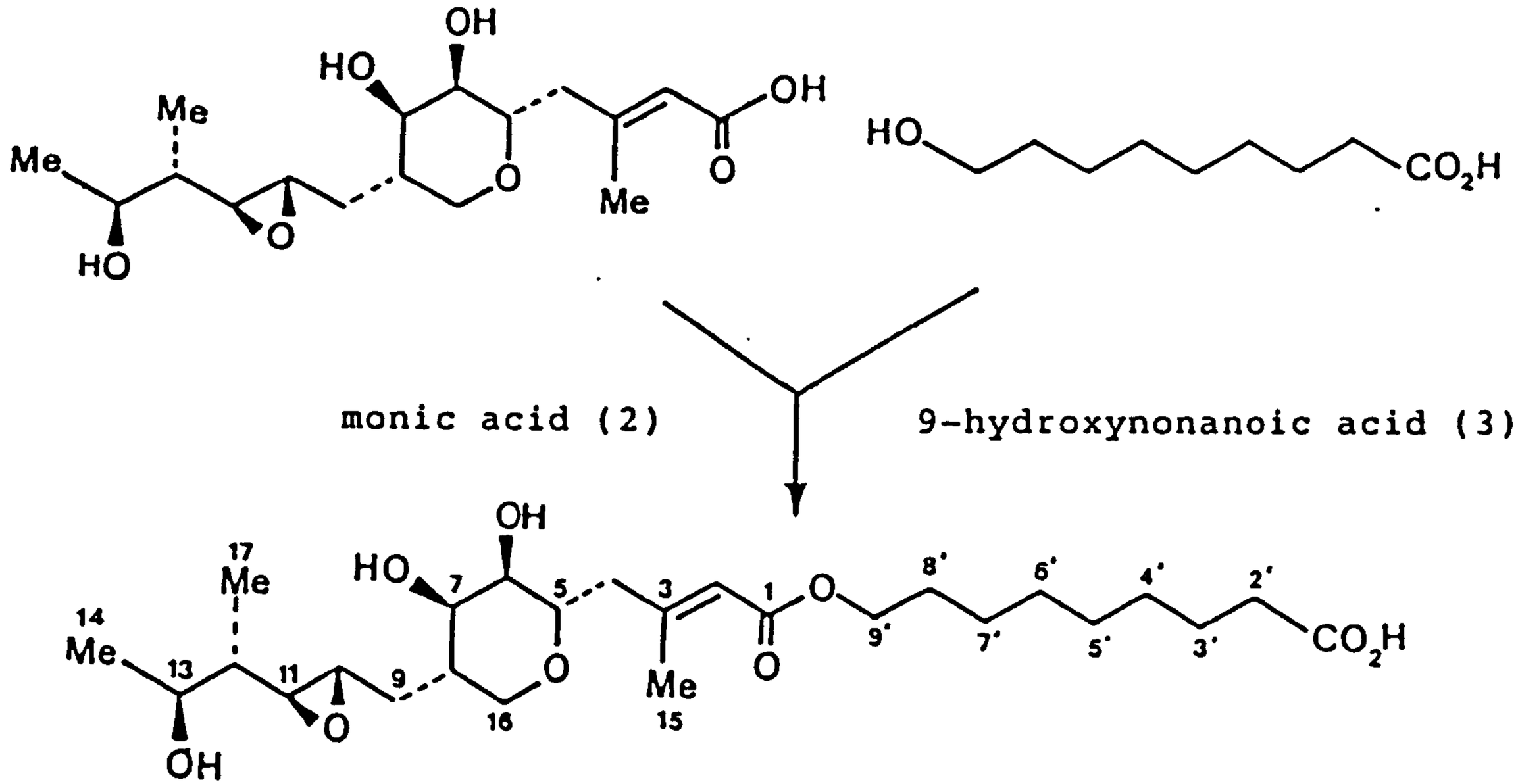
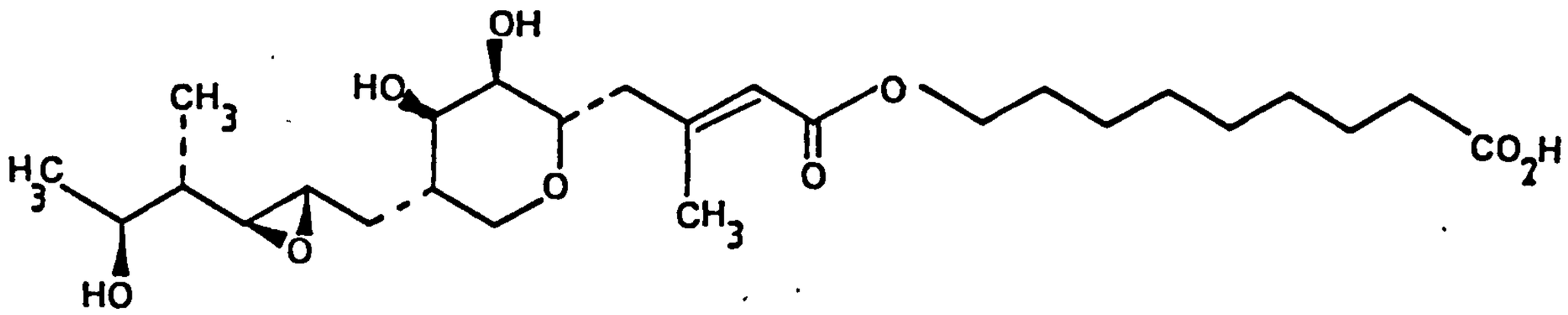
CHAPTER ONE

Introduction

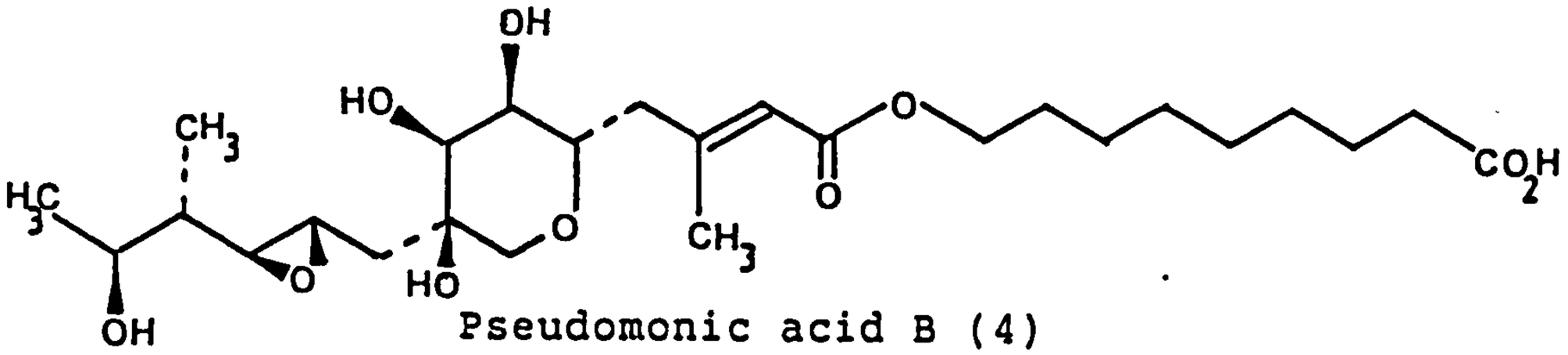


Pseudomonic Acid A (1)

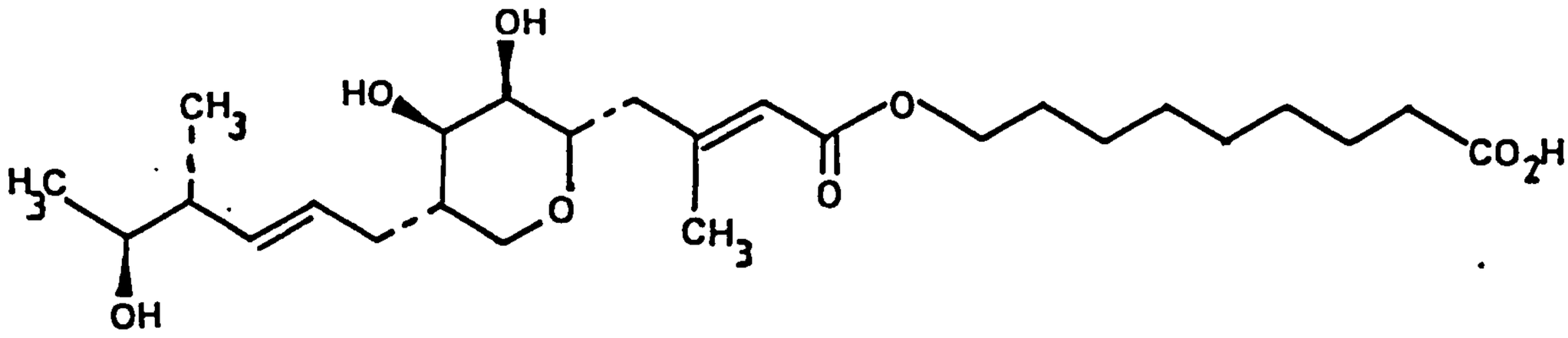
Scheme 1.1



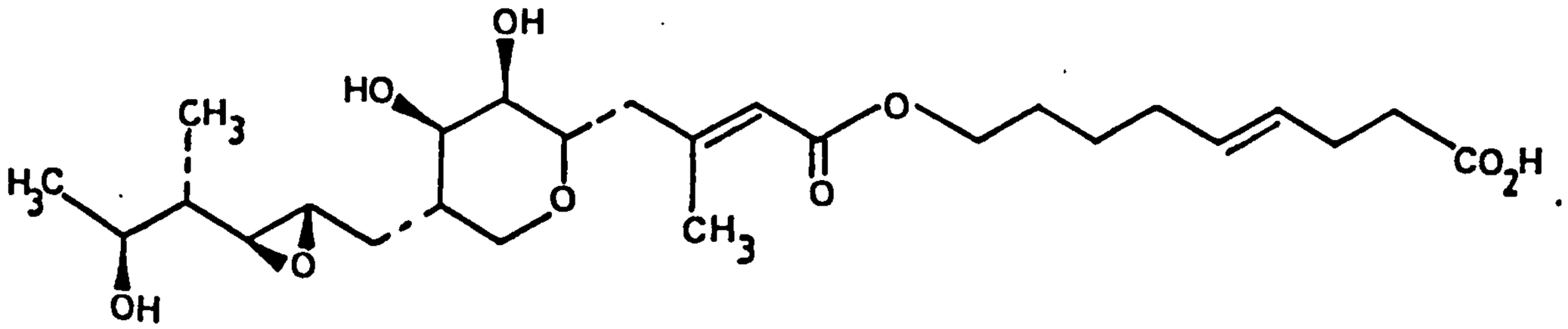
Pseudomonic acid A (1)



Pseudomonic acid B (4)



Pseudomonic Acid C (5)



Pseudomonic Acid D (6)

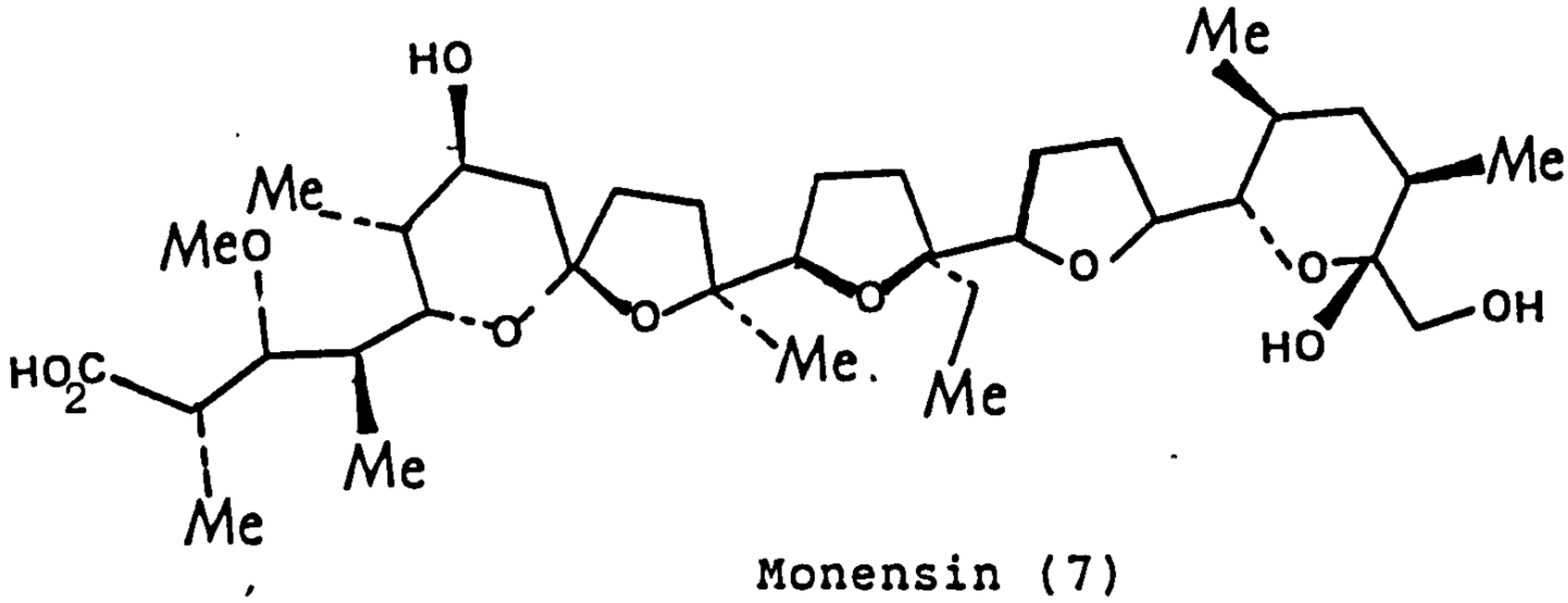
Introduction

1.1 CHEMISTRY AND STRUCTURE

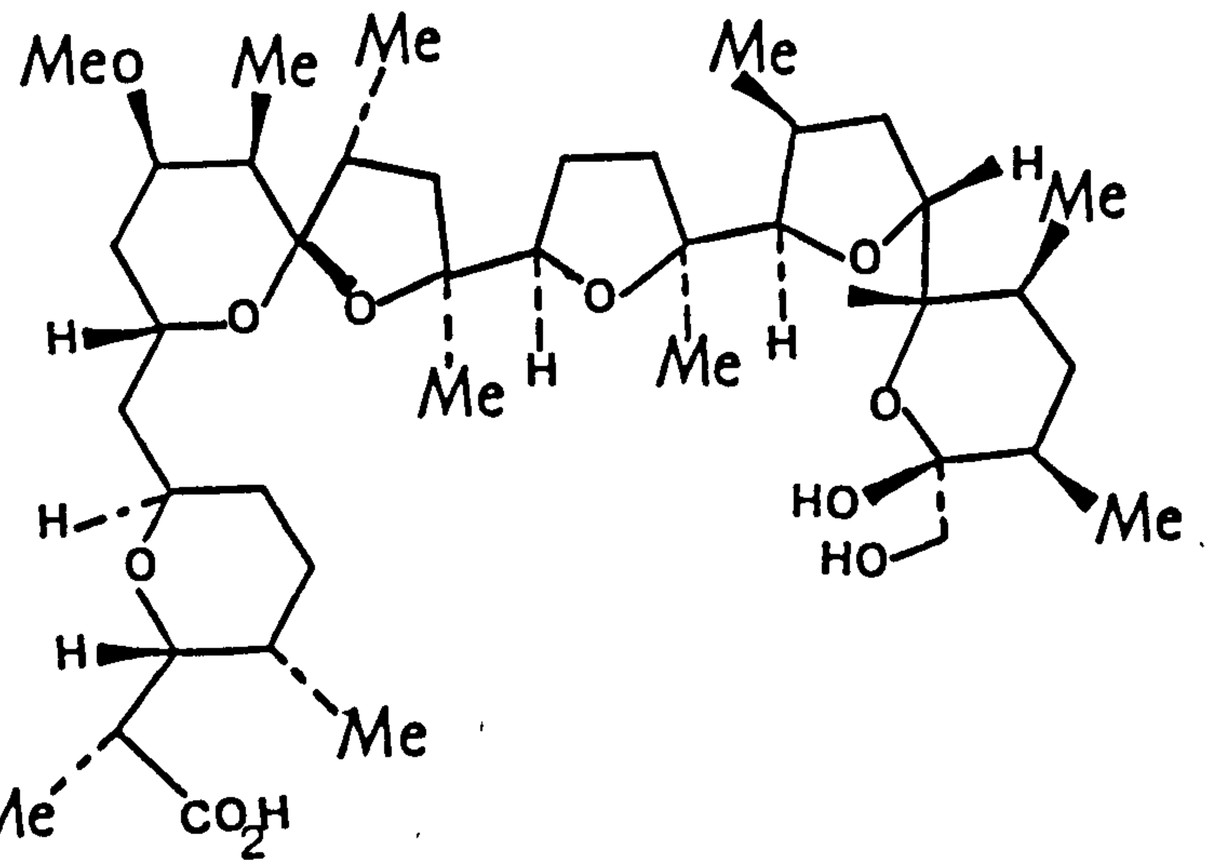
Fermentation of *Pseudomonas fluorescens* (NCIB 10568) produces a group of novel antibiotics collectively known as the pseudomonic acids[1]. They are now being used clinically under the generic name "mupirocin" for the topical treatment of skin infections. The therapeutic value of these antibiotics has been clinically developed by Beecham[2]. They function as competitive inhibitors of isoleucyl-t-RNA synthetase[3] and are effective antimicrobical agents against Gram positive bacteria and mycoplasmal pathogens. Cross resistance with other commonly used antibiotics is not observed and they are also effective against multiple strains of *Staphylococcus aureus* .

Detailed studies of their chemical and structural characteristics have been fully reported in a series of papers[4]. The major metabolite, pseudomonic acid A (1) has a complex structure consisting of: a C_{17} unsaturated carboxylic acid moiety (2), known as monic acid, containing epoxide, diol and tetrahydropyran ring functions which has been esterified by an unique 9-hydroxynonanoic acid moiety (3), as shown in scheme 1.1.

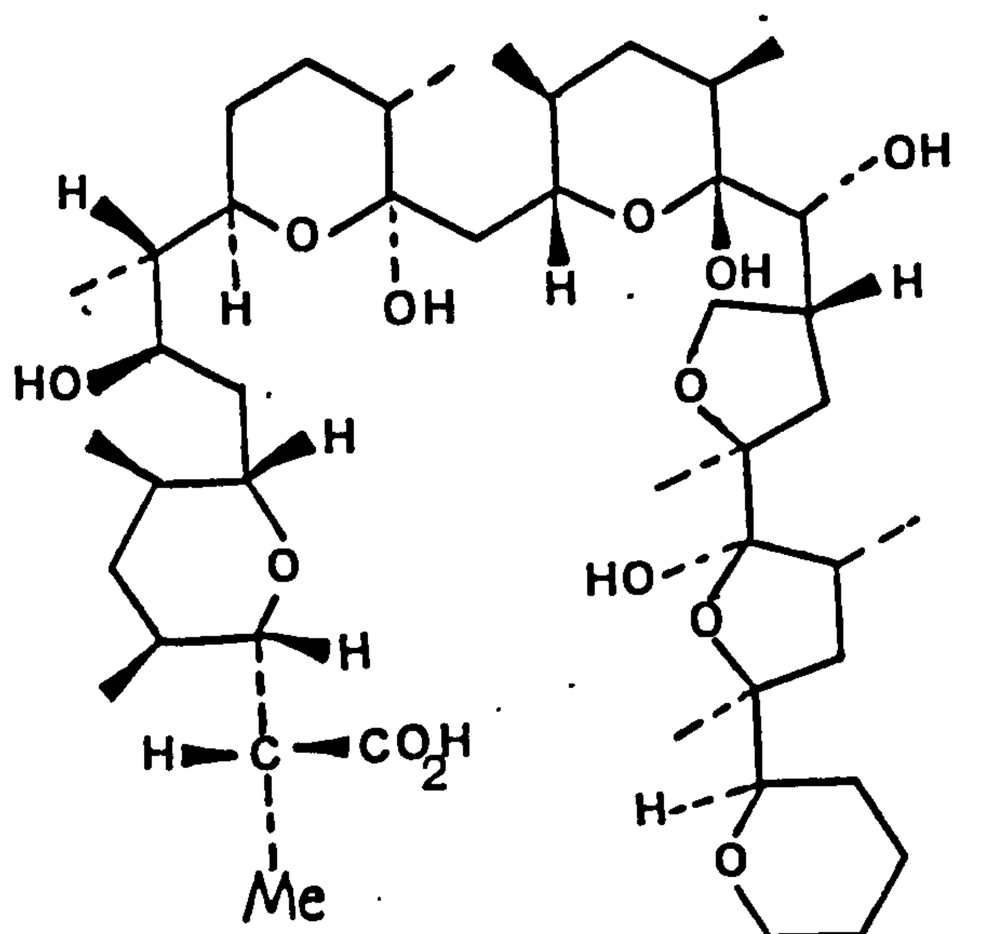
The minor metabolites, designated pseudomonic acids B (4), C (5), and D (6), all possess this basic nucleus of monic acid (2) but differ in that B contains an extra hydroxyl group at



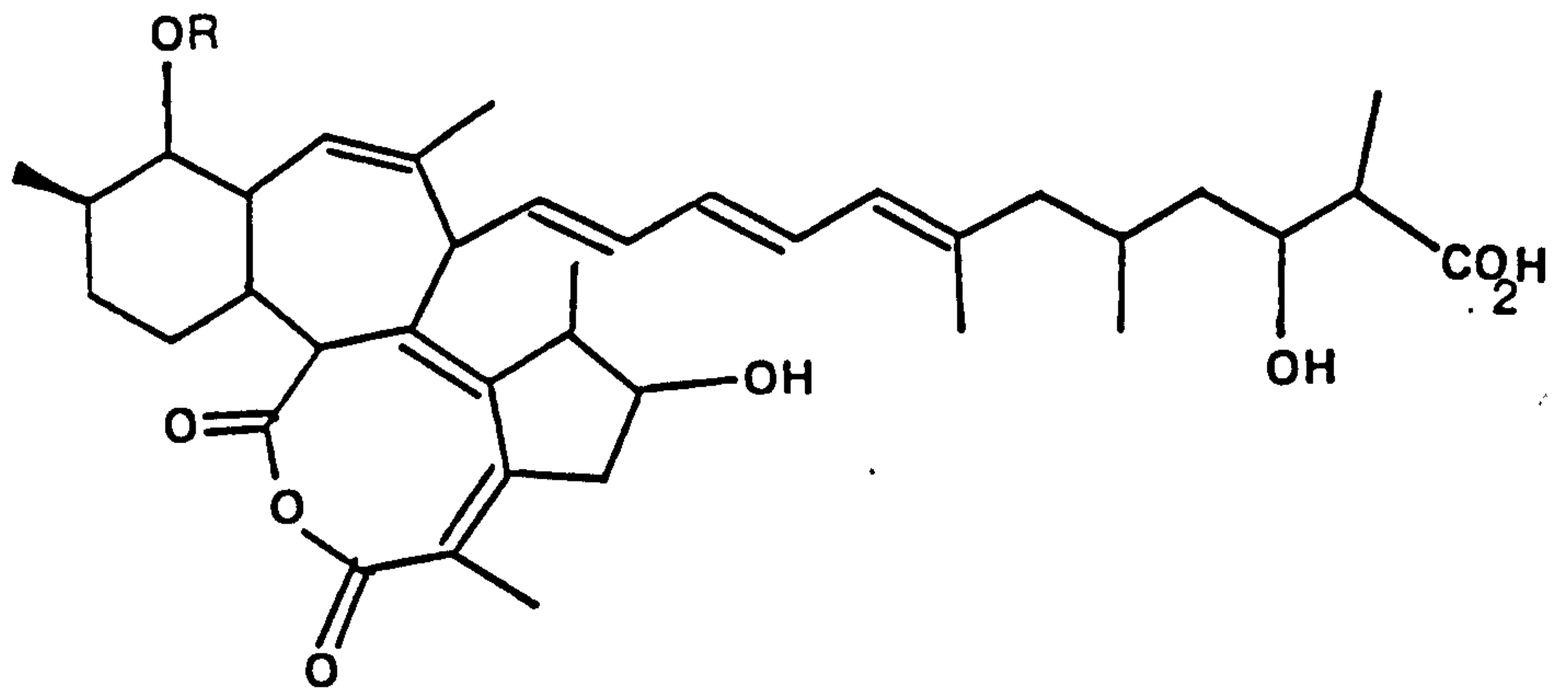
Monensin (7)



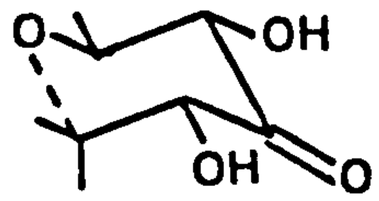
Nigericin (8)

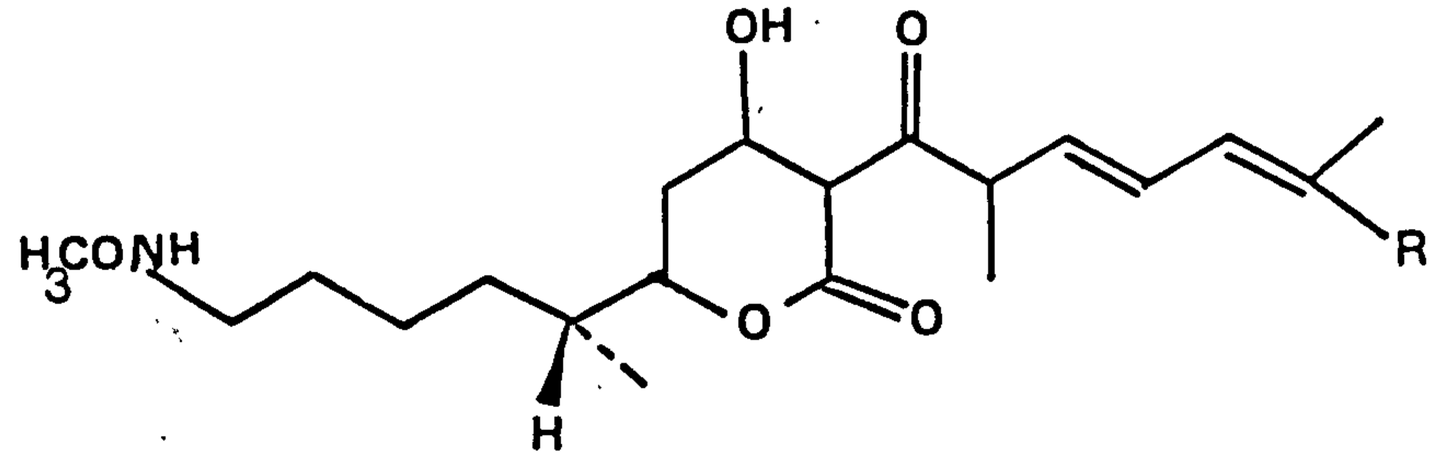


Alborixin (9)



Aurantinin A (10) R=H
Aurantinin B (11) R=



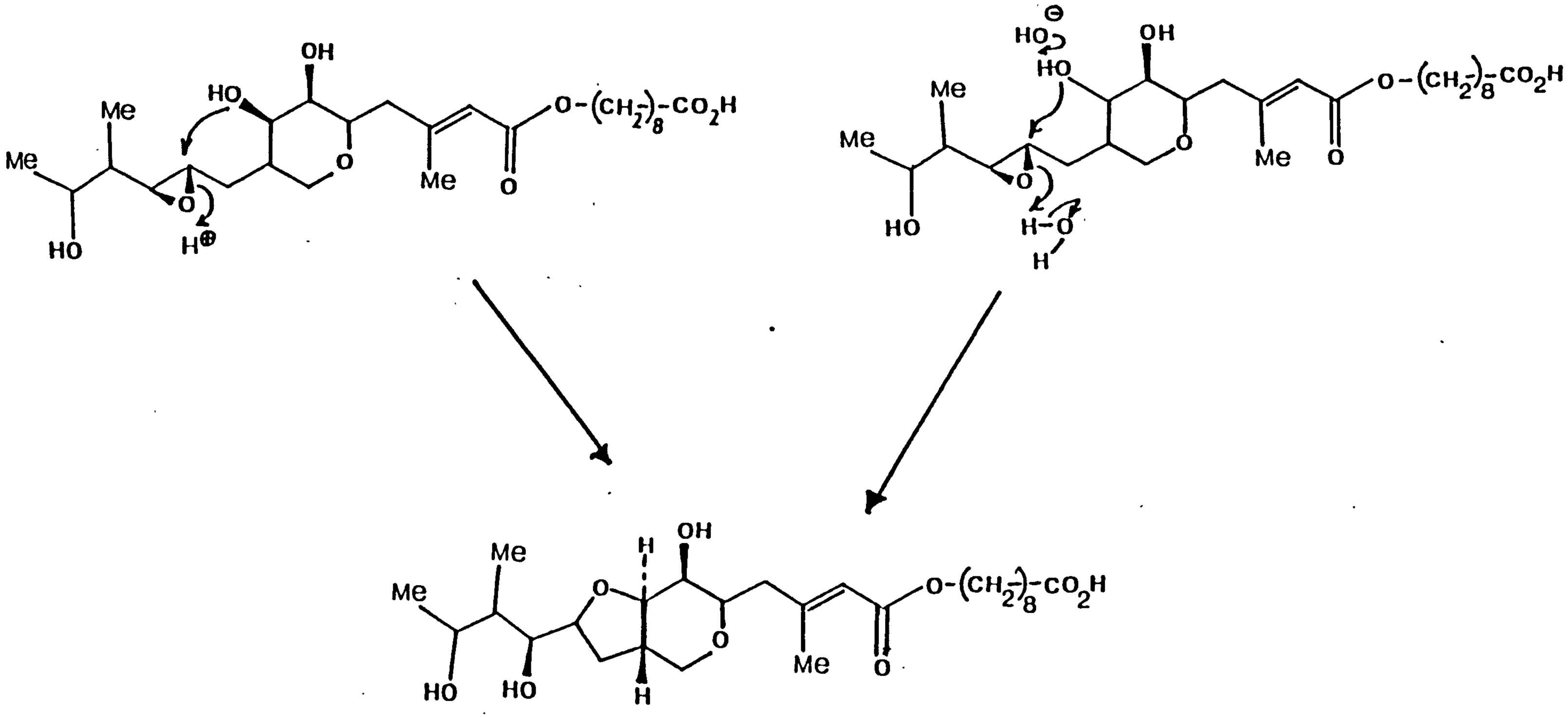


Myxopyronin A (12) R=$CH_2CH_2CH_3$
Myxopyronin B (13) R=$(CH_2)_3CH_3$

C-8; C has a trans carbon-carbon double bond in place of the epoxide at C-9 and D has a trans carbon-carbon double bond at C-4′ in the 9-hydroxynonanoic acid side chain.

Classification on a structural basis with any of the known antibiotic groups[5] is not really possible. The nearest relatives of pseudomonic acid are perhaps, the polycyclic polyether monocarboxylic acids such as monensin (7), nigericin (8) and alborixin (9). These compounds function as antibiotics by influencing cation transport across membranes. Other bacterial metabolites which possess antibacterial antibiotic activity are, for example, the aurantinins A and B[6] (10) and (11). Like pseudomonic acid they are polyketide derived with the basic biological precursors being acetate and methionine. Again, the presence of a methyl group formed by decarboxylation of acetate is found and is common with pseudomonic acid and the myxopyronines A and B[24] (12) and (13) which are all true bacterial metabolites.

Chain and Mellows[1] observed that the antibiotic activity of pseudomonic acid A in solution was dependent on pH. Activity was retained within the range pH 4-9 but was gradually lost outside these limits. The acid and base catalysed rearrangement products derived by intramolecular opening of the epoxide ring, have been shown to be the trans-fused bicyclic structures[7,8] (14) and (15). Scheme 1.2, illustrates the mechanisms for the formation of these rearrangement products. Hence, these compounds are isomeric with pseudomonic acid A. Their full ^{1}H and ^{13}C nmr



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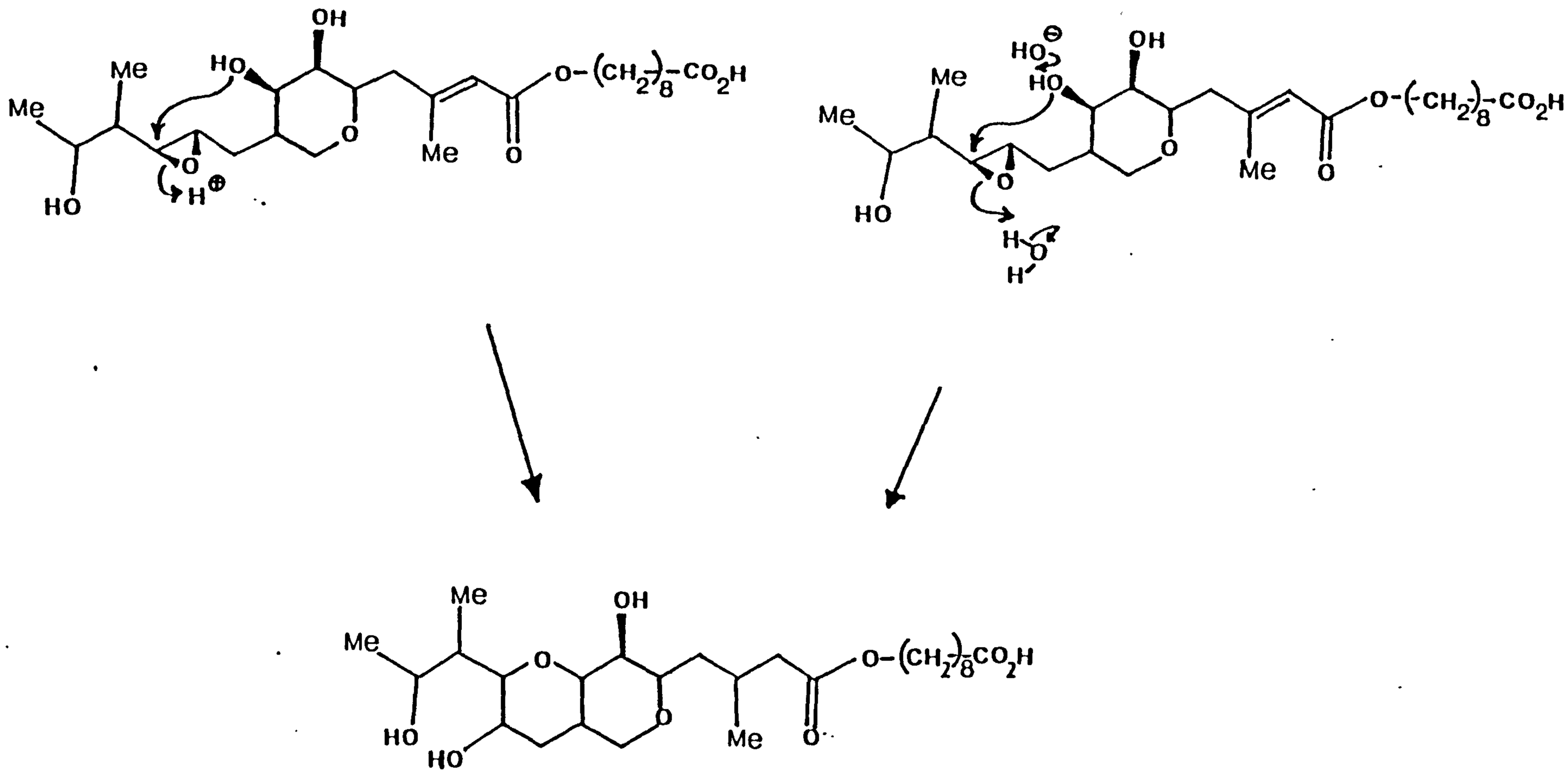
Scheme 1.2 (cont)



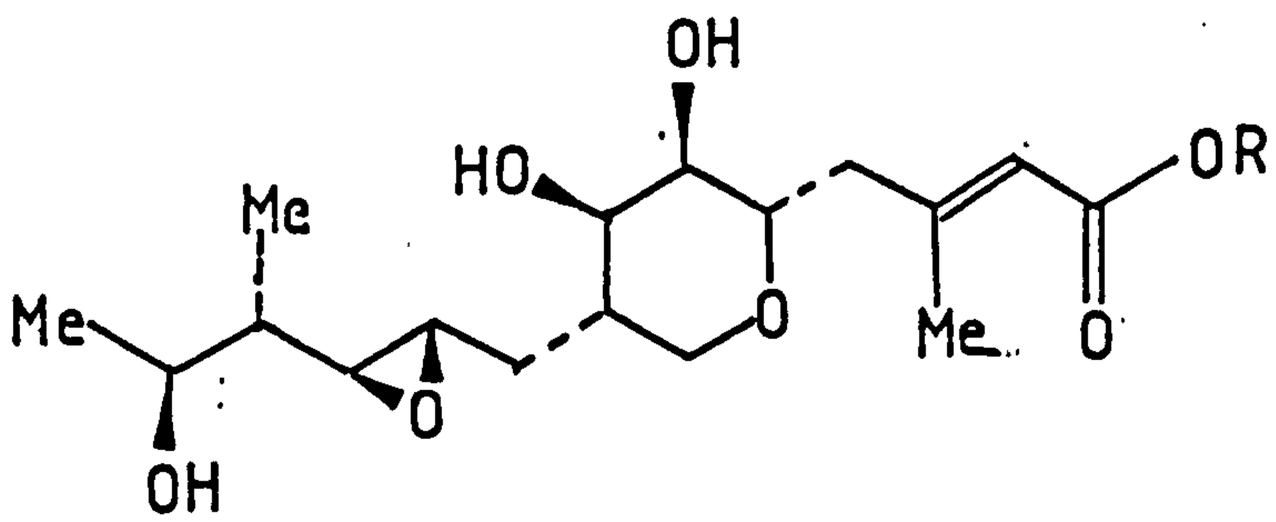
(14)

Scheme 1.2

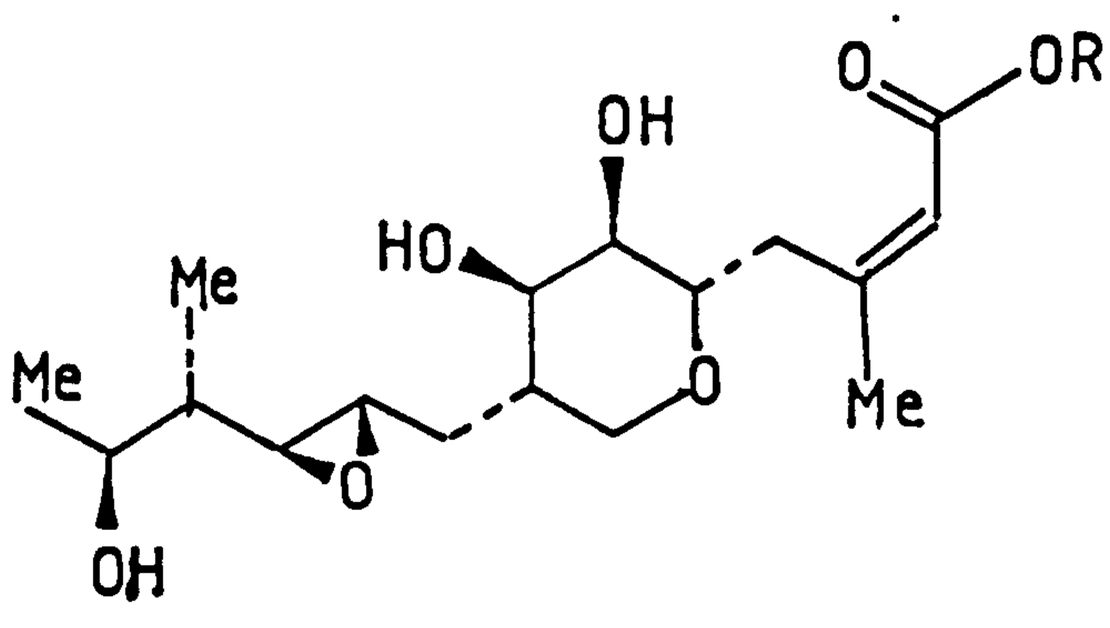
assignments have been published recently[9]. In view of the interest in pseudomonic acid as a novel antibiotic, this chemical instability to acid and alkali has been investigated[7]. It was found that in acid solution (pH 1.0) rearrangement was complete within 30 minutes. However, in alkaline solution (pH 13.0) rearrangement was much slower with only 30% rearrangement observed after 30 minutes. It was complete after 18 hours.

No interconversion between these two isomeric compounds was observed when these rearrangement products were subjected to the same acidic and basic conditions of the rearrangement reactions. Therefore, these rearrangement isomers are derived from monic acid by two competing mechanisms in which the relative rates of intramolecular rear side attack of the 7-hydroxy group on the epoxide carbons 10 and 11 are dependent on the reaction conditions. In these studies, monic acid was used to avoid complications arising from the hydrolysis of the unsaturated ester.

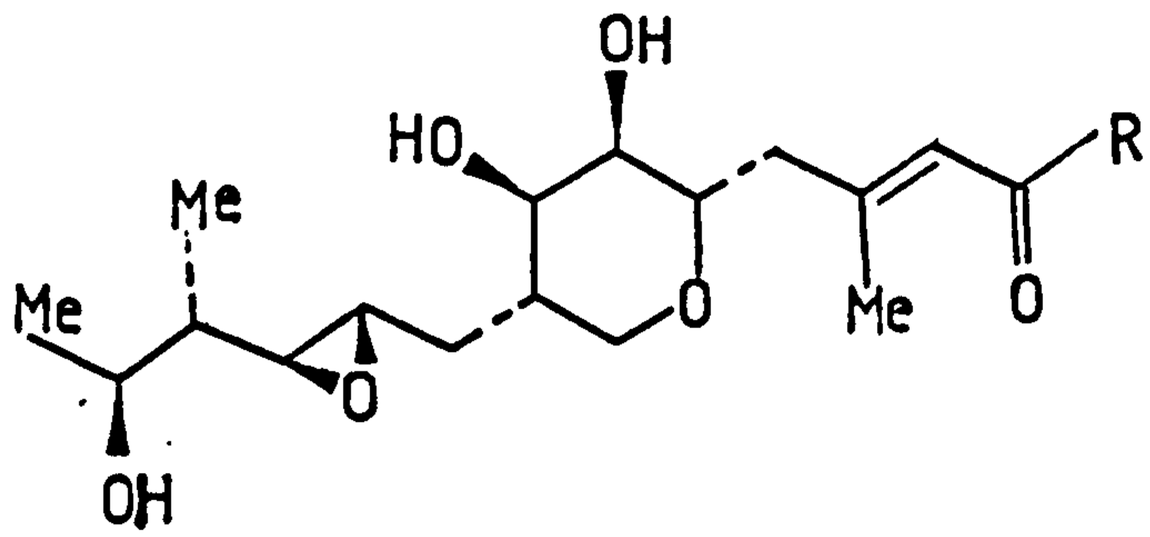
The effect of chemical modification[2,4,8] on the biological properties of pseudomonic acid has also been investigated. When given systemically to mammalian species, including man, it is rapidly metabolised with loss of antibacterial activity. Another unfavourable characteristic of pseudomonic acid is its degree of binding to serum protein. It binds to the extent of 95% to human serum protein and since it is only the unbound portion of the antibiotic that is free to exert any antibacterial action, chemical modification to try to reduce this high degree of binding



(2) R=H
(16) R=Me



(17) R=Me
(18) R=$(CH_2)_8CO_2Me$



(19) R=Me
(20) R=Et_n
(21) R=Bu^n

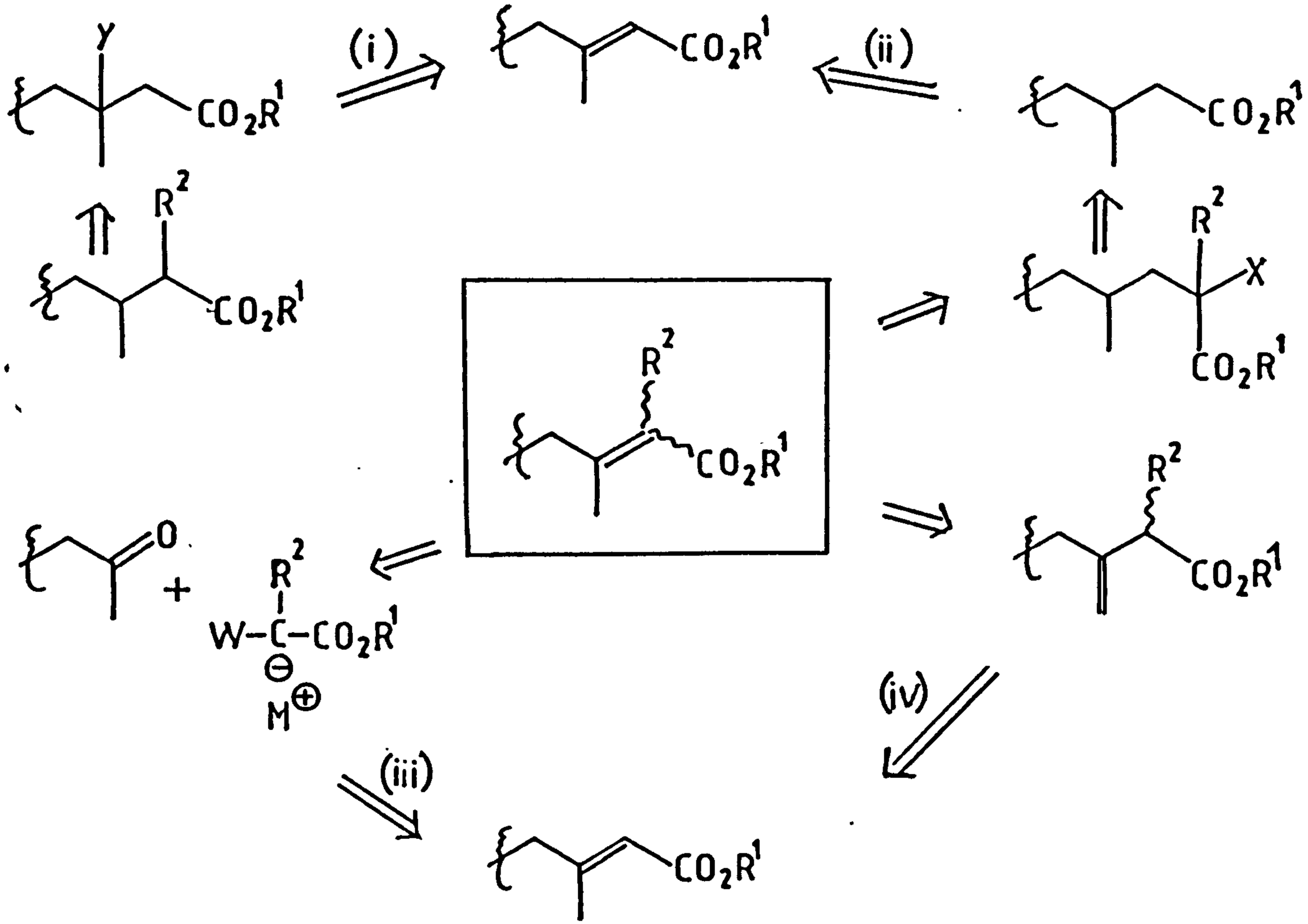
was investigated.

In general, the degree of protein binding appears to correlate with lipophilicity. Hence, one approach used was to reduce the lipophilicity of pseudomonic acid by replacing the 9-hydroxynonanoic acid side chain with shorter chain alcohols. Methyl monate (16) was significantly less protein bound (30% bound) and it was also antibacterially active with a spectrum of activity similar to that of pseudomonic acid. The un-natural isomers, methyl iso-monate (17) and methyl iso-pseudomonate (18) were 100-fold less active than the corresponding natural trans forms.

Interestingly, monic acid (2) which is the nucleus of all the pseudomonic acids exhibits no antibacterial activity[7]. With suitable protection and deprotection, the 9-hydroxynonanoate side chain can be cleaved and the resulting monic acid nucleus can then be used to generate a wide variety of esters. Apart from the esters mentioned previously none of these synthetic analogues showed antibacterial activity.

The biological properties of αβ-unsaturated ketones[4(d)], prepared from monic acid have also been investigated. Only three ketones (19), (20) and (21) of the group prepared displayed interesting antimicrobial activity.

To try to reduce the rate of metabolic cleavage of the allylic ester, effects of substitution at C-2 was investigated[2]. Alkyl, halogen and a variety of other


Y
CO_2R^1
(i)
CO_2R^1
(ii)
CO_2R^1
R^2
CO_2R^1
R^2
X
CO_2R^1
R^2
CO_2R^1
R^2
CO_2R^1
O
+
R^2
$W-\overset{|}{C}-CO_2R^1$
$\ominus$
$M^{\oplus}$
(iii)
CO_2R^1
(iv)

Scheme 1.3

substituents were tried. The synthetic approaches to these analogues are outlined in scheme 1.3.

(i) Addition of X-Y to an allylic ester followed by displacement of X then elimination of H-Y.

(ii) Hydrogenation, alkylation and electrophilic substitution with X^+ followed by elimination of H-X.

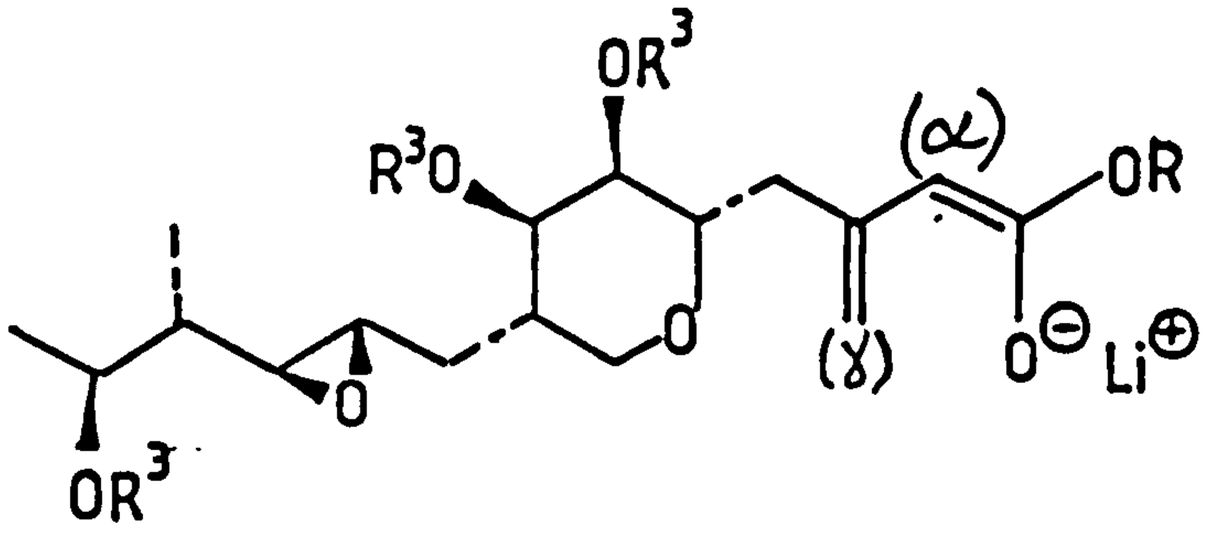
However, neither of these approaches were successful.

(iii) Peterson olefination works.

(iv) Electrophilic addition to dienolates. Both of these approaches work and are of practical value.

Anions derived from 2-substituted phosphonoacetates generally did not react except for ethyl 2-fluoro phosphonoacetate, which gave good yields of ethyl 2-fluoromonate and its isomer. Anions of α-substituted silyl esters react efficiently with the ketone in route (iii) but stereoselectivity was found to be highly in favour of the biologically inactive isomonate esters. Antimicrobial activity was found in only the 2-fluoro and the 2-methyl monate esters.

Additional work on route (iv)[4(d)] was used to insert substituents at C-15 as well as at C-2. This was achieved by use of lithium dienolates (22). The hydroxyl groups of monic acid were first protected as trimethylsilyl ethers. Electrophilic addition can occur at either the 'hard' nucleophilic α position (C-2) or at the 'soft' nucleophilic γ-position (C-15). The regiochemistry is thus dependent on



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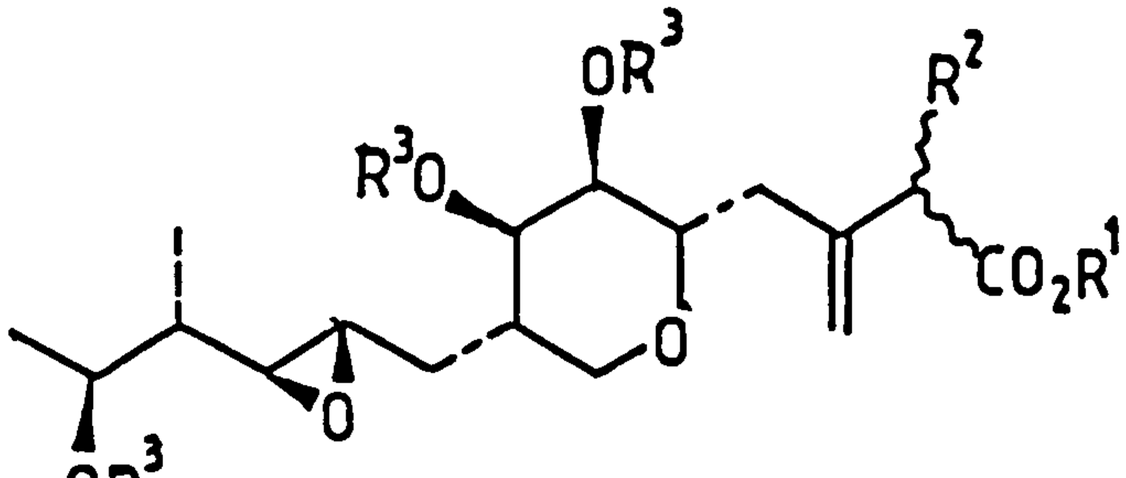

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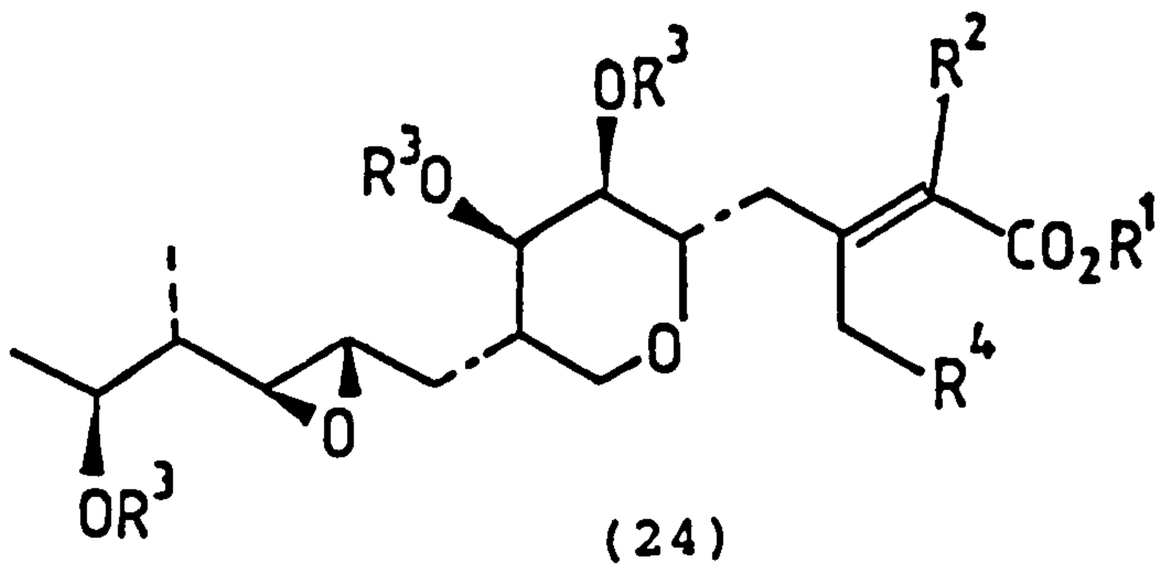

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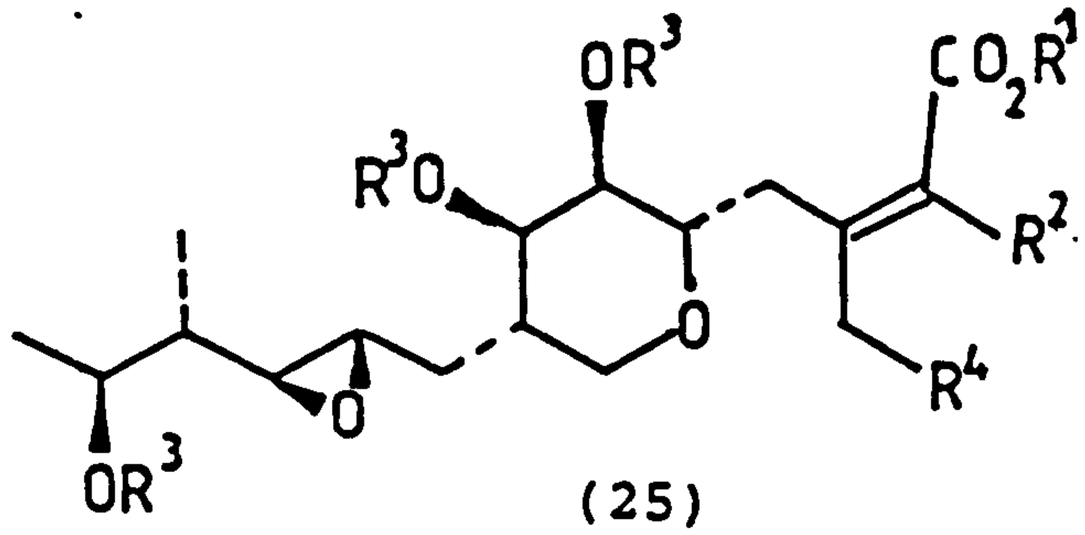


(25)

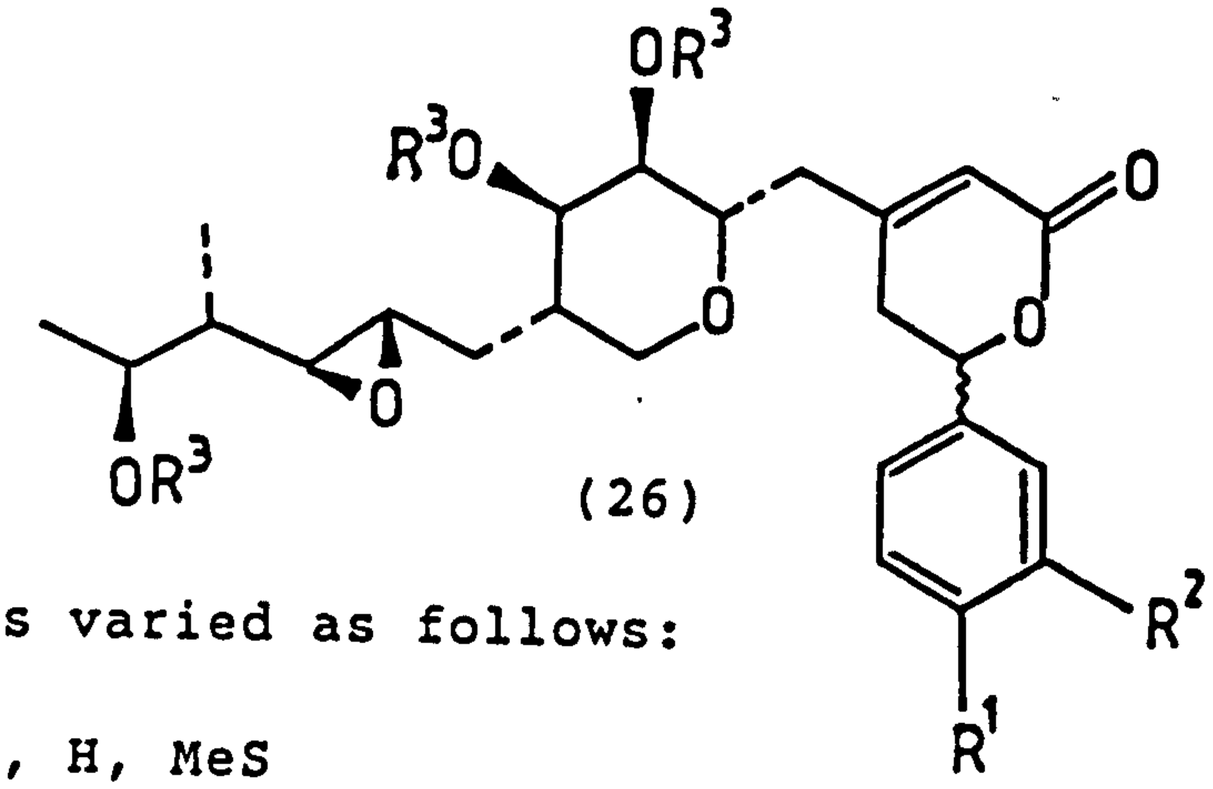
R groups varied as follows:

R_1= Me, Et

R_2= H, Me, Et, PhS, MeS, $PhCH_2$, D

R_3= H, $SiMe_3$

R_4= H, D, $SiMe_3$, PhS, PhSe



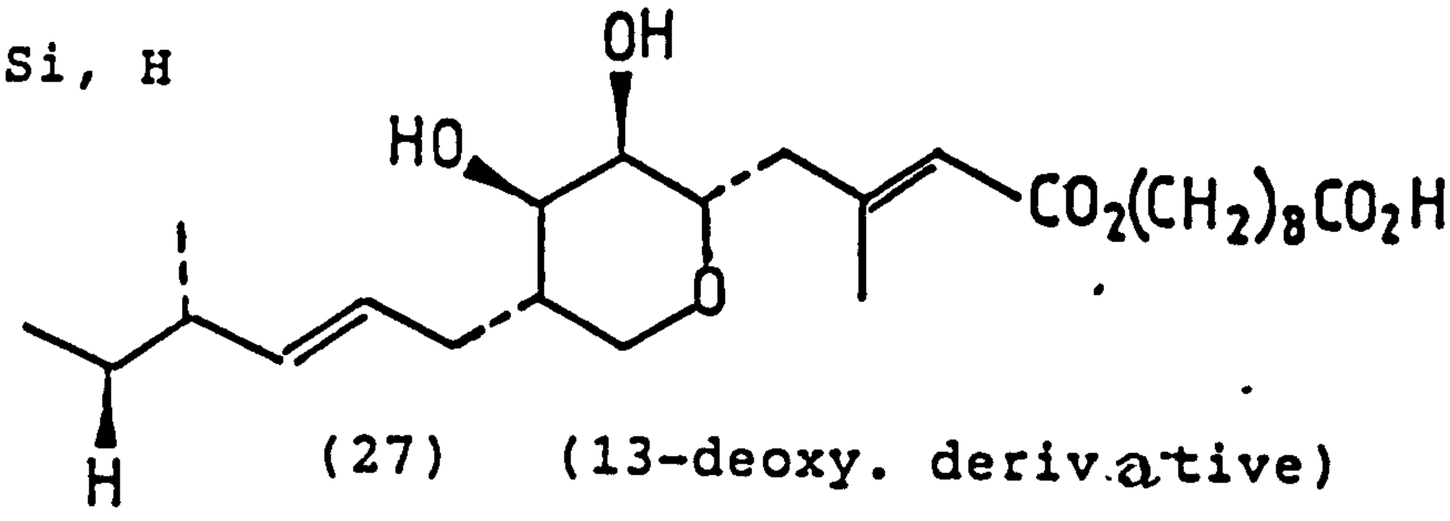
(26)

R groups varied as follows:

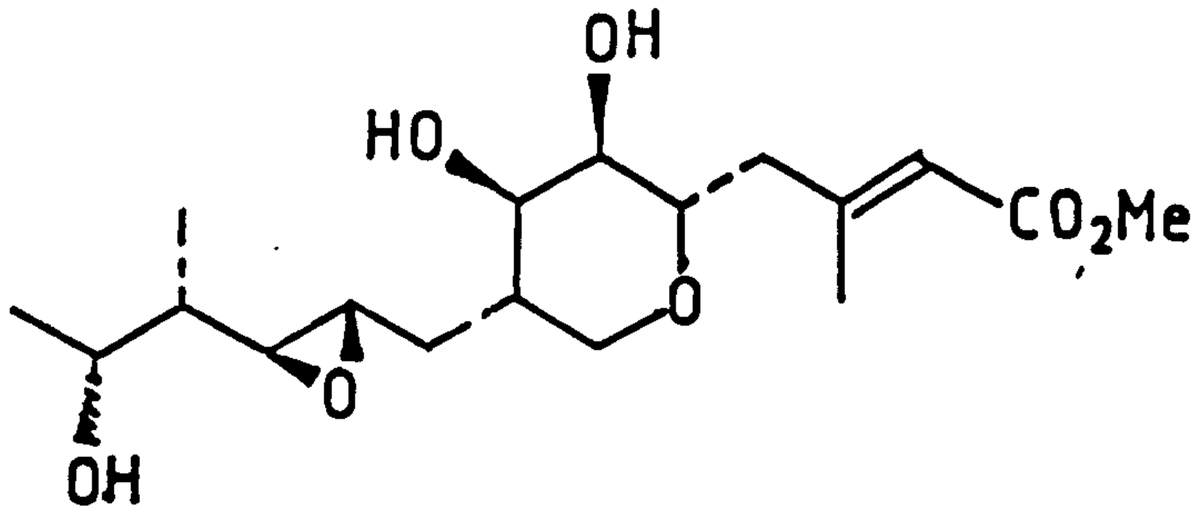
R_1= MeO, H, MeS

R_2= NO_2, CN, H

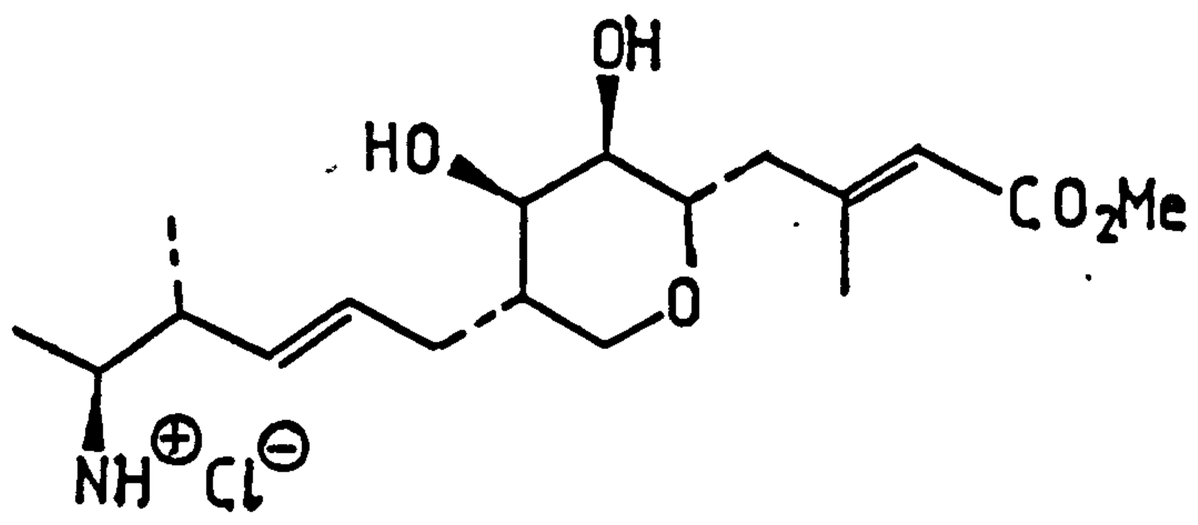
R_3= Me_3Si, H



(27) (13-deoxy. derivative)



(28) (inversion of chiral centre at C-13)

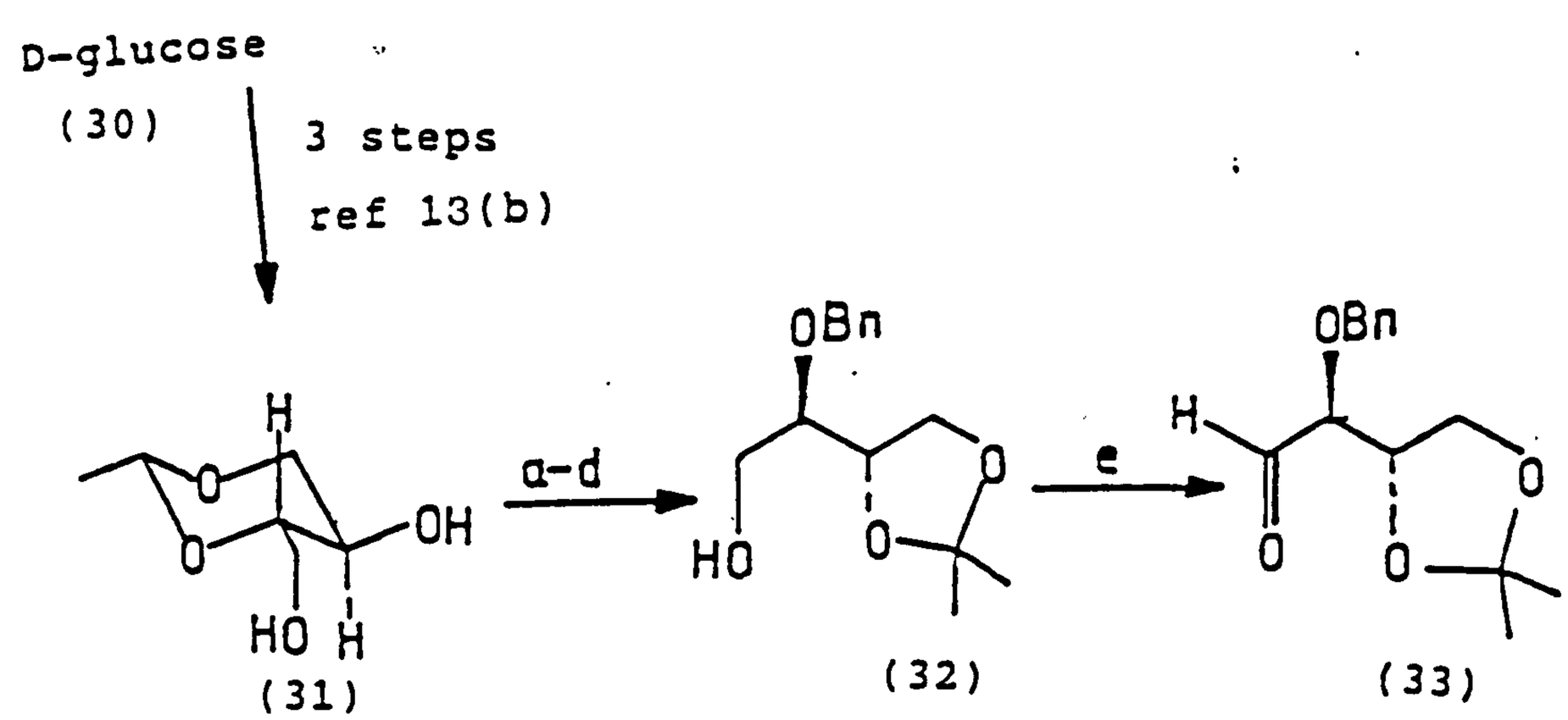


(29) (13-amino. derivative)

the nature of the nucleophile. Addition to the α-position provides the diastereoisomeric deconjugated esters (23) whilst addition to the alternative γ-position affords mixtures of the conjugated esters (24) and (25) in varying ratios according to the electrophile.

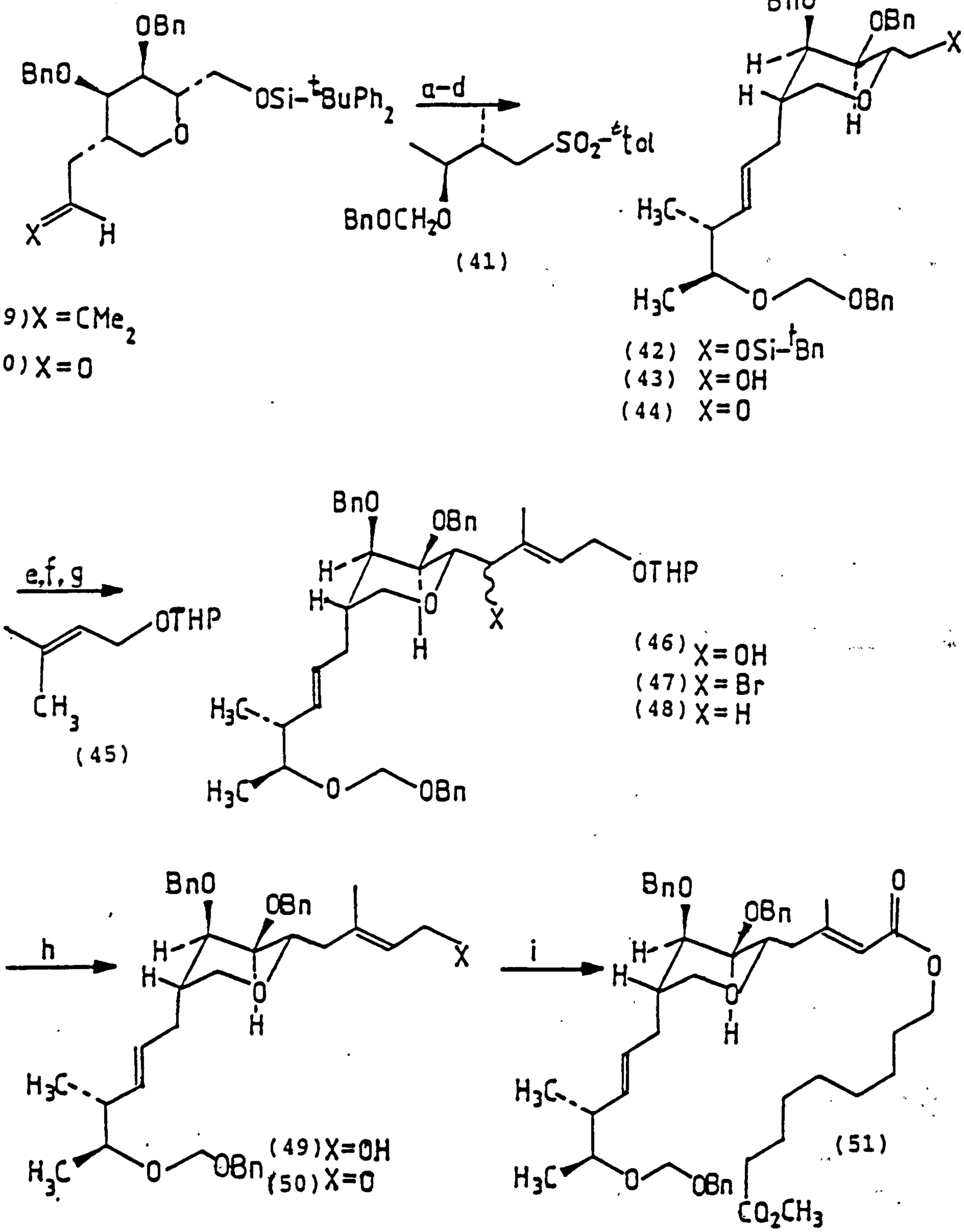
Results of this study, showed that only esters of 2-fluoromonic acid and 2-methylmonic acid possessed significant antibacterial and antimycoplasmal activity. Esters of 2-fluoromonic acid offered no advantages in metabolic activity. However, esters of 2-methylmonic acid did show significant increases in stability in tissue homogenates and *in vivo* over analogous esters of monic acid. No antimicrobial activity was observed from either the deconjugated esters or the dihydropyrones (26) prepared from them.

Finally, a report of more recent work[10], on the effects of chemical modification of the pseudomonic acids details the effects of reduction, inversion and replacement of the C-13 hydroxyl group, with an amino group. It was known the deoxy deriviative (27), could not be formed in the presence of 10, 11 epoxide and so was replaced by a carbon-carbon double bond. Also, for the formation of the amine deriviative, it was necessary to carry out the transformation on the pseudomonic acid C series because the reduction step, oxime to amine, also resulted in the reduction of the ester and epoxide functions. The results of this study were that the 13-deoxy compound (27), methyl (13R)-monate (28) and the 13-amino-derivative (29) displayed little antimicrobial



Scheme 1.4

Reagents: (a) Trityl chloride (1.1 equiv), DMAP (1.1 equiv), DMF (80%) (b) NaH dispersion, THF, benzyl bromide (83%) (c) 40% conc. HCl in methanol, 22°C, 14h (84%) (d) PPTs (0.1 equiv), acetone, 18h, (78%) (e) ClCOCOCl (1.5 equiv), Me_2SO (2.4 equiv), CH_2Cl_2, -78°C; then Et_3N (2.4 equiv), -78°C→0°C (87%) (f) 9-BBN triflate, Et_2O, thio ester 33 (1 equiv), diisopropylamine (1.4 equiv); add 32 at -78°C→0°C (79%) (g) $LiAlH_4$, ether, 0°C→22°C, 1h (85%); TsCl (1.4 equiv), CH_2Cl_2, DMAP (1.1 equiv), Et_3N (2.5 equiv), 22°C, 4h (i) TsOH (1.1 equiv), MeOH, 16h, 22°C; add $NaOCH_3$ in methanol (80% yield of 36).



Scheme 1.5

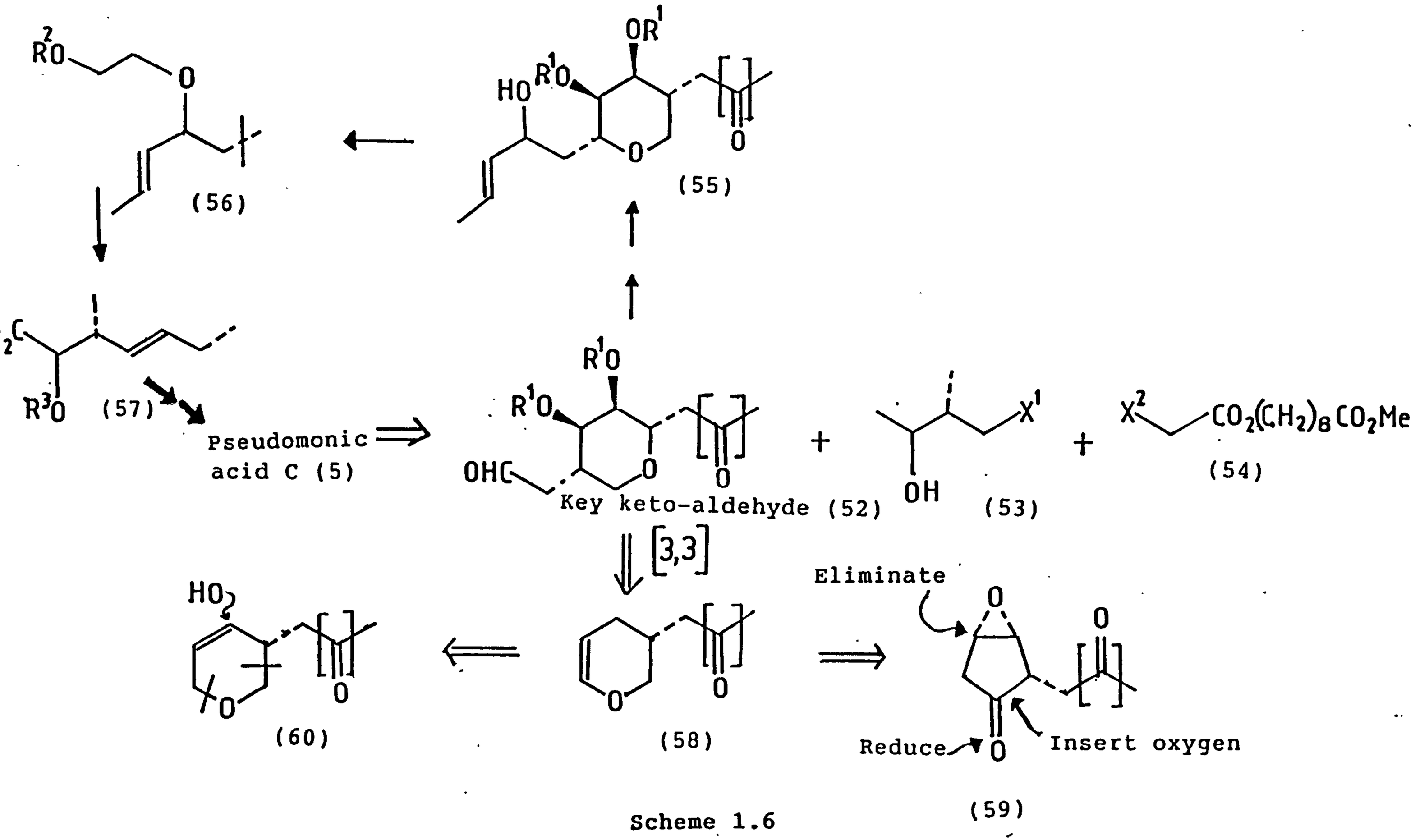
(a) Formation of carbanion 40; LDA, (1.1 equiv), THF, -60°C; addition of CS_2 (excess), -60°C→ +5°C, 20min; add CH_3I (10 equiv), 22°C, 1h, (96%); (b) $^nBu_3SnH$ (1.5 equiv), toluene, cat. AIBN, 95°C (86%); 1.0M $^nBu_4N^+F^-$ in THF (85%); PDC (1.7 equiv), CH_2Cl_2, 3Å powdered molecular sieves (76%); (e) preparation of 44 at -100°C; add anhydrous CeI_3 (1 equiv) in THF, 30min; add 43, -100°C→ 0°C; quench aq. NH_4Cl (65%); (f) CBr_4, CH_2Cl_2, $BaCO_3$ (1.7 equiv), Ph_3P (1.7 equiv), 45min (75%); (g) Bu_3SnH (1.5 equiv), toluene, AIBN, 65°C, 1h, (82%); (h) PPTs (0.5 equiv), MeOH, 22°C, 14h (98%); (i) MnO_2 (excess), THF, 5h; then methyl 9-hydroxynonanoate, NaCN, HOAc, 16h (98%).

activity.

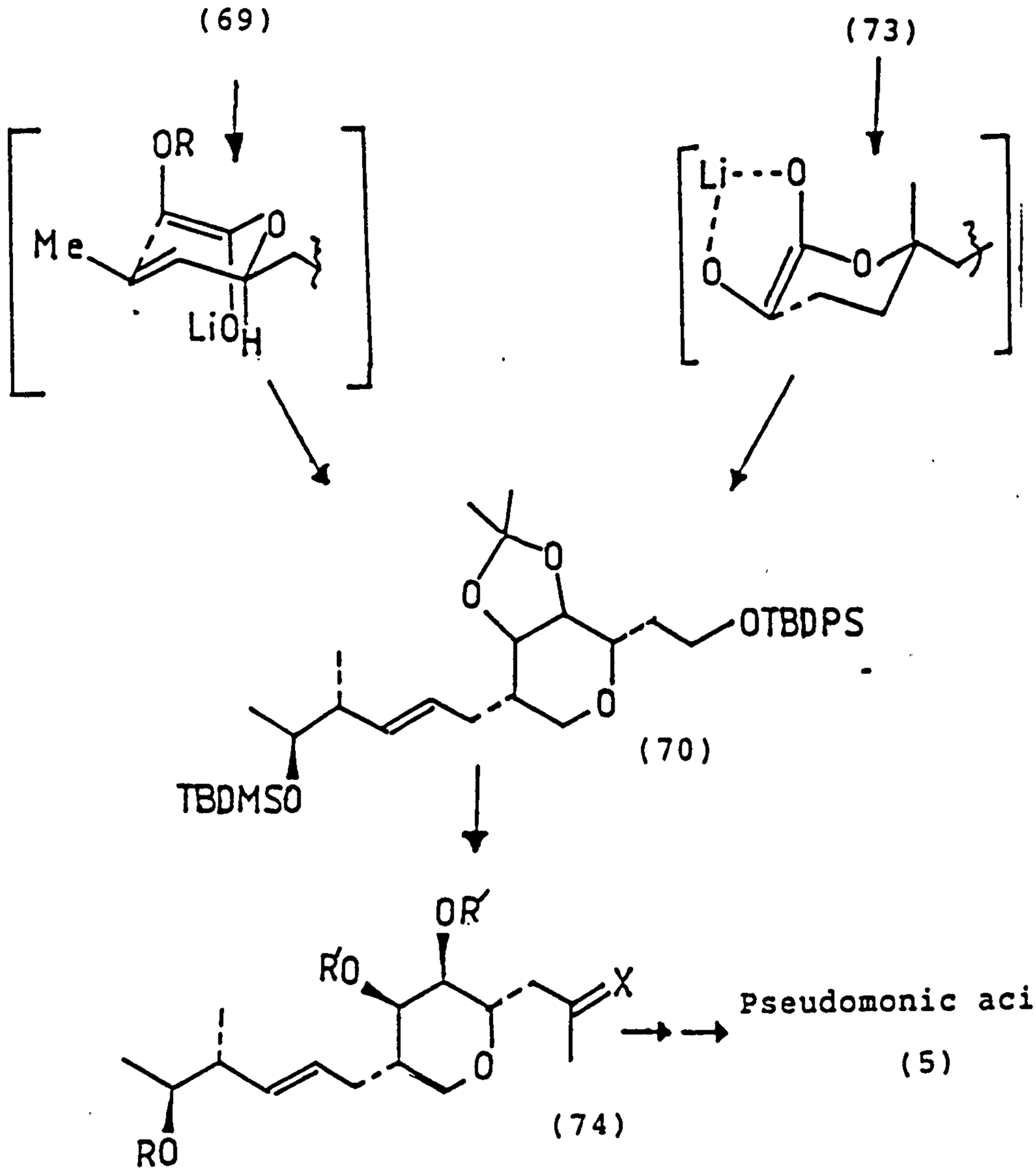
1.2 SYNTHESIS

Notwithstanding a narrow range of activity constrained mainly to Gram positive bacteria, the good activity of the pseudomonic acids against various skin pathogens combined with a novel and challenging structure have made them inviting targets for total synthesis. There are a large number of complete or formal syntheses of these compounds in the literature[11]. The conversion of pseudomonic acid C (5) to the major metabolite, pseudomonic acid A (1), has also been reported[12].

Almost exclusively, these synthesis have been directed toward the A or C series. Moreover, while some are enantioselective, most of these routes rely on sugar derivatives as a source of asymmetry. For example, in the synthesis of pseudomonic acid C[13] the chiral tetrahydropyran, bearing 4 contiguous asymmetric centres, suggested the use of a reduced carbohydrate precursor. The necessary absolute configuration at C-5 is the same as that found in the series of L-hexoses. Scheme 1.4 shows how the desired absolute stereochemistry at C-5 as well as at C-6 was introduced at the start of this synthesis from the chiral starting material, D-glucose[13b] (30). Further transformations to allow for attachment of the carbons 11 - 14 of pseudomonic acid C and subsequent conversion to the natural product are shown in scheme 1.5.

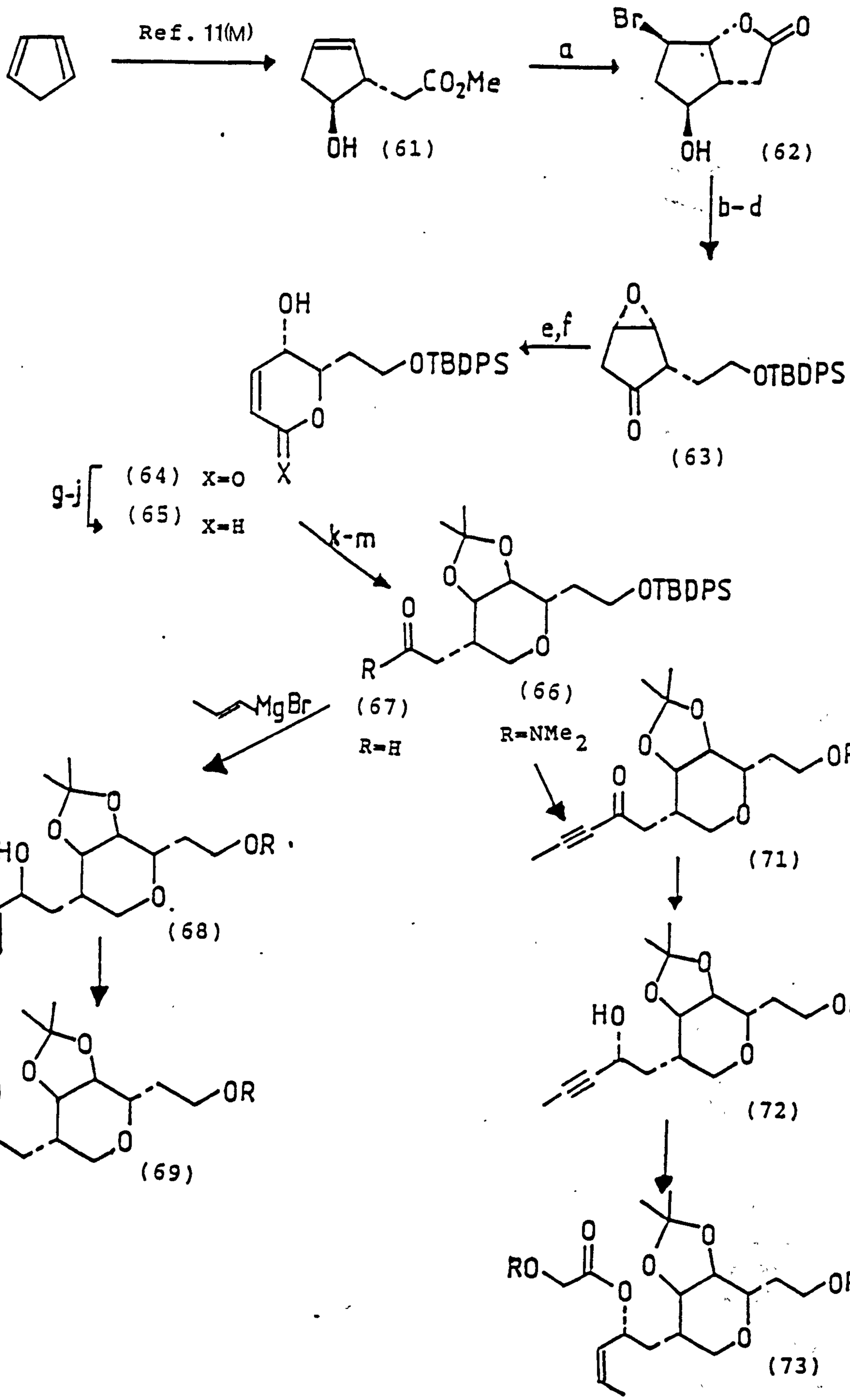

R2O
O
(56)
OR1
HO
R1O
O
O
(55)
MeO2C
R3O
(57)
R1O
Pseudomonic
acid C (5)
R1O
OHC
O
O
Key keto-aldehyde (52)
X1
OH
(53)
X2
CO2(CH2)8CO2Me
(54)
[3,3]
HO
O
O
(60)
(58)
Eliminate
O
O
Reduce
O
Insert oxygen
(59)

Scheme 1.6



Scheme 1.7 (cont)

Reagents and conditions: (a) KOH, H_2O, then NBS, THF (62%) (b) $LiBH_4$, Et_2O, then 15% NaOH (100%) (c) $tBuPh_2SiCl$ (88%) (d) PCC (95%) (e) m-CPBA (f) Et_3N (74% from 63) (g) $tBuMe_2SiOTf$ (90%) (h) Dibal (i) Et_3SiH, $BF_3.Et_2O$, CH_2Cl_2, -78°C (55% overall), (j) HOAc, THF, H_2O (3:1:1) (75%), (k) $MeC(OMe)_2-NMe_2$, xylene (95%), (l) OsO_4 (cat), NMO (90%) (m) CH_3COCH_3, p-TosOH (90%) (n) propenylmagnesium bromide, $ZnBr_2$, 1 equiv. THF, -78°C (75%); HPLC separation of isomers (o) t-$BuMe_2SiOCH_2CO_2H$), DCC, DMAP (81%); Me_3SiCl then LDA *in situ* Me_3SiCl, THF, -78°→25°C (55%) esterfication (R'=t-$BuMe_2Si$) (p) conversion of methyl ester to C-14 methyl group; Dibal (80%); I_2, PPh_3 (75%); Bu_3SnH, AIBN, EtOH, 25°C (70%), (R'= t-$BuMe_2Si$ (q) propynyl lithium, $BF_3.Et_2O$, THF, -78°C (96%) (r) (R)-alpineborane, THF, 25°C (88%) (s) $PhCH_2OCH_2CO_2H$, DCC, DMAP (93%); then H_2, 10% Pd/$BaSO_4$, pyridine, 25°C (86%) (t) LDA, THF, -78°→25°C; then Me_3SiCl (60%); then esterification (R'= Bn). Completion of the synthesis, conversion to methyl ketone then Horner-Emmons condensation [$(MeO)_2P(O)CH_2CO(CH_2)_8CO_2Me$, NaH, THF, 25°C (80%) and deprotection to pseudomonic acid [HOAc, 25°C (76%)].


Ref. 11(M)
CO2Me
OH
(61)
a
Br
O
O
OH
(62)
b-d
OH
OTBDPS
O
X
e,f
O
OTBDPS
O
(63)
g-j
(64) X=O
(65) X=H
k-m
O
O
O
OTBDPS
R
O
(66)
MgBr
(67)
R=H
R=NMe2
HO
OR
O
(68)
RO
O
O
OR
O
(69)
O
O
O
OR
O
(71)
HO
O
O
OR
O
(72)
RO
O
O
O
OR
O
(73)

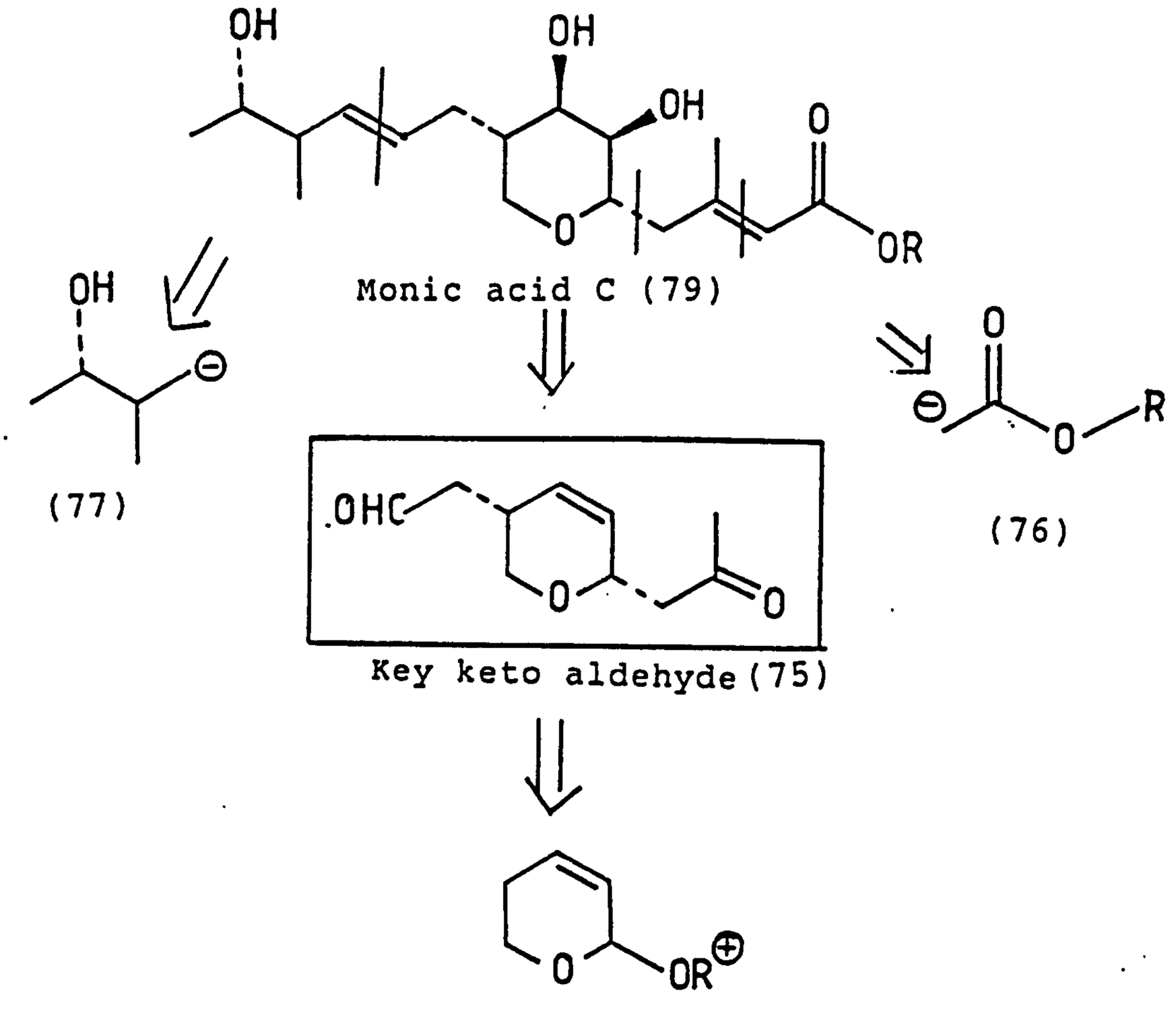
Scheme 1.7

Another conceptually new approach to the total synthesis of pseudomonic acid C^{11m}, employed the use of a modified glycolate ester enolate Claisen rearrangement to introduce the required stereochemistry.

In most of the previous syntheses, the simplifying disconnection at the 2,3 and the 10,11 double bonds of pseudomonic acid C to give the keto-aldehyde (52) were made. Conversion back to the natural product was then achieved in a convergent manner by Wittig-Horner or Julia coupling (scheme 1.6).

However, in this synthesis (scheme 1.7), the side chain stereochemistry at C-12 and C-13 was related back to the chirality around the pyran ring. The use of a silicon protecting group on the glycolate carbonyl ester inhibits chelation between the ether and carbonyl oxygens. If deprotection is performed under strictly kinetic conditions, the required threo (anti) ester is formed rather than the usual (*E*) selective deprotonation. This route should also be amenable to the preparation of analogues of the pseudomonic acids.

Further work on this route has now been published[14]. The key intermediate, aldehyde (52), has been made from simple starting materials and the side chain stereochemistry was developed by using the chirality of the central pyran fragment. However, the silyl-based glycolate ester enolate Claisen rearrangement proceeded with only moderate levels of stereoselectivity.

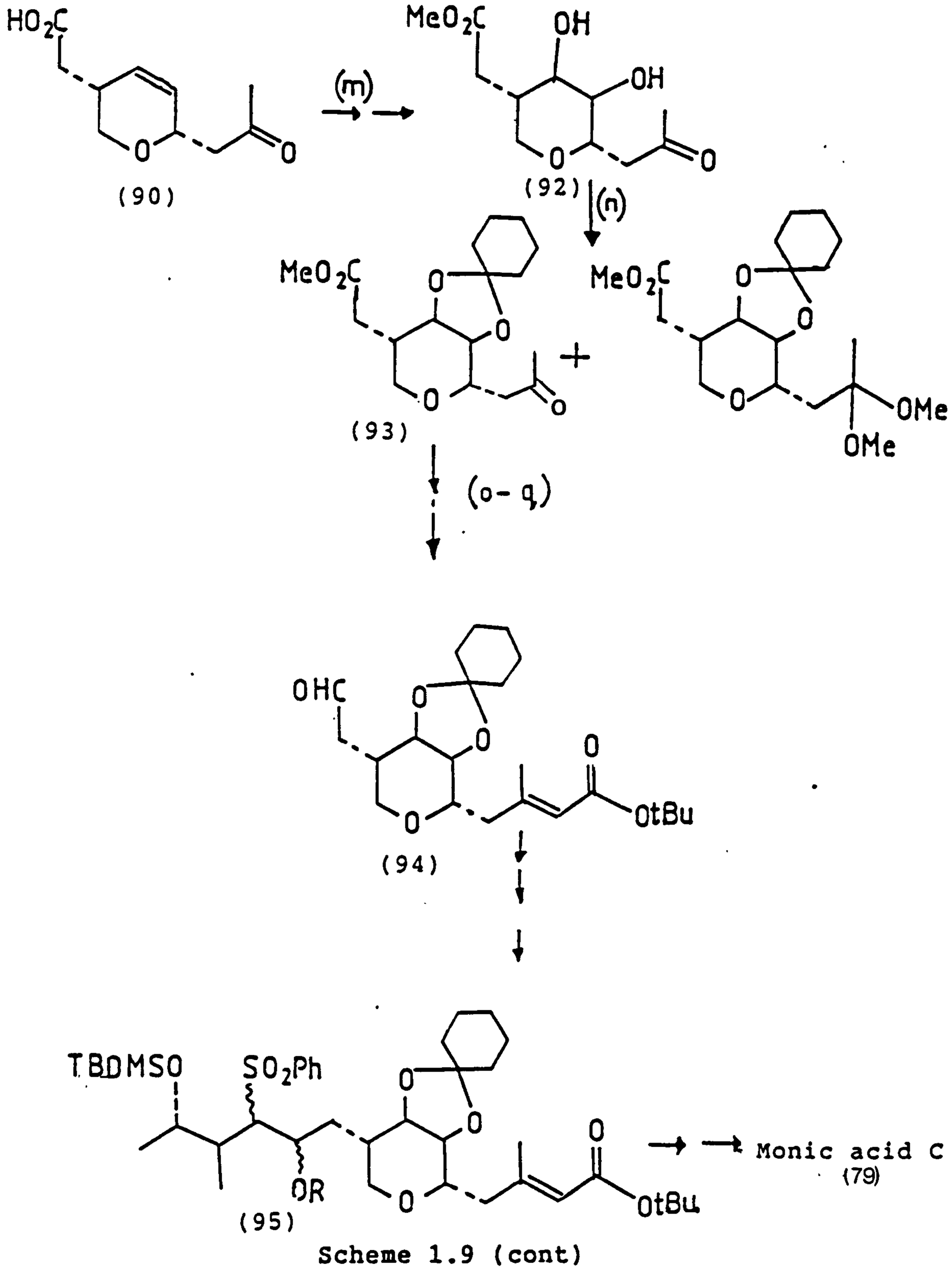

OH
OH
O
O
OR
Monic acid C (79)
OH
(77)
O
R
(76)
OHC
O
O
Key keto aldehyde (75)
O
OR⊕
(78)

Scheme 1.8

From a practical standpoint, a synthesis of non-racemic pseudomonic acid C is especially attractive since this substance is chemically stable and retains its antibacterial activity over a broad pH range. Whereas pseudomonic acid A shows rapid loss of activity outside the range pH 4-9, a property associated with intramolecular attack on the epoxide by the C-7 hydroxyl substituent, as discussed above. In the quest for such a route, the stereoselective total synthesis of (-)-monic acid C (79) has been investigated[15].

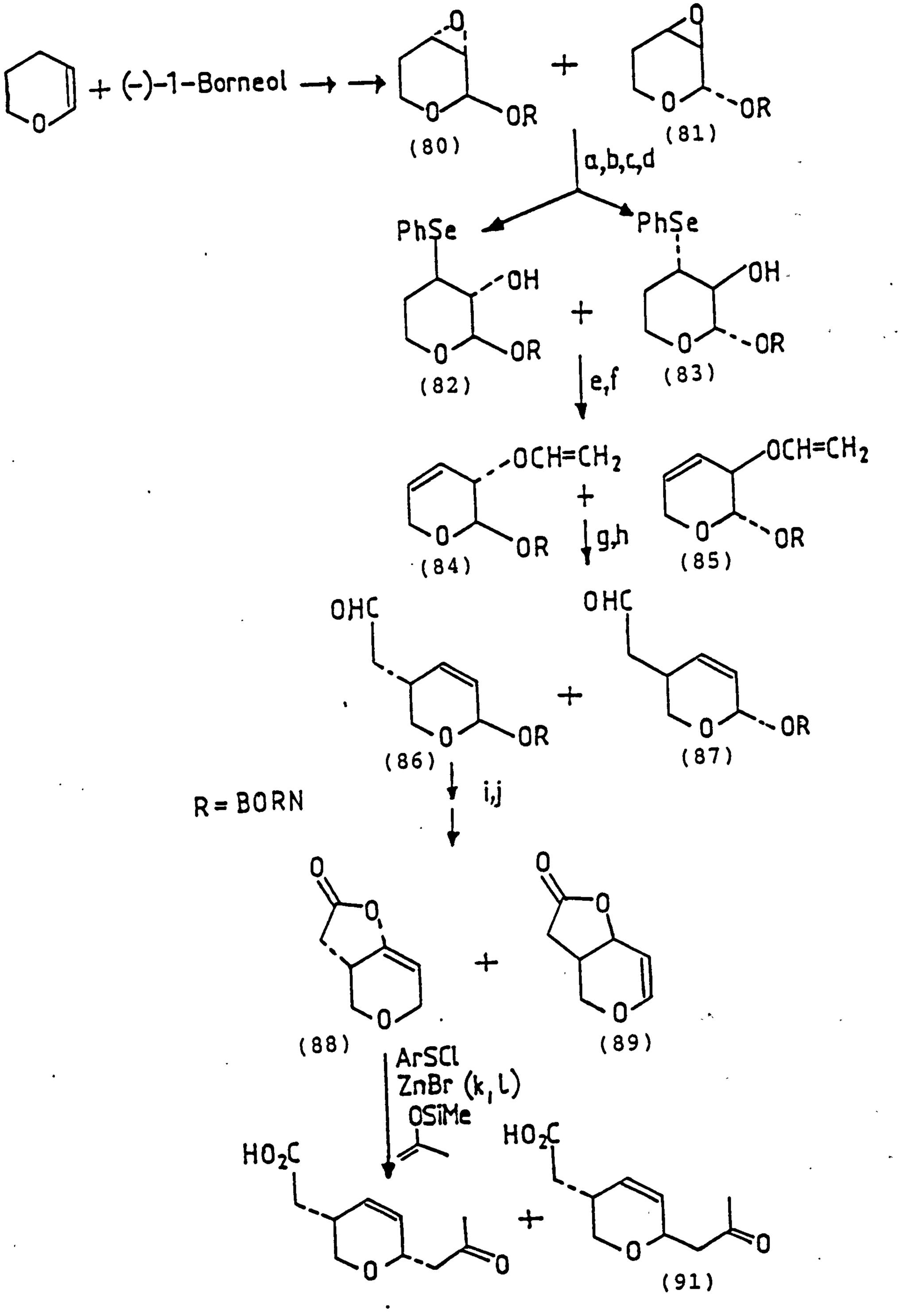
This synthesis has been accomplished in a linear sequence of 22 steps beginning from dihydropyran and with (-)-1-borneol as a chiral auxiliary. This was required since the synthesis starts from an achiral starting material (dihydropyran) and so an enantioselective step is required if optical resolution is to be avoided.

The synthetic strategy adopted is depicted in scheme 1.8. The absolute stereochemistry of the key keto-aldehyde (75) is established in this route through an asymmetric functionalisation of a dihydropyran (78) using (-)-borneol as the chiral auxiliary. The convergence of this key intermediate (75) with a five-carbon segment (77) derived from ethyl (2S,3S)-3-hydroxy-2-methylbutanoate , and a three-carbon species (76), derived from acetone, sets all six chiral centres of monic acid C (79) in place. Scheme 1.9 shows how this dihydropyran was then elaborated via trans epoxides (80, 81) to hydroxyselenides (82, 83). These were then converted to vinyl ethers (84, 85) which then underwent Claisen rearrangement to give (86, 87). Carbosulfenylation



Scheme 1.9 (cont)

Reagents: (a) Br_2, $C_6H_5NMe_2$, CH_2Cl_2, $-78^oC \rightarrow 0^oC$, 6h (b) DBU, 95^oC, 20h (c) m-$ClC_6H_4CO_3H$, CH_2Cl_2, 48h (d) PhSeSePh, $NaBH_4$, LiBr, EtOH-THF, $0^oC \rightarrow 40^oC$, 3h (f) $CH_2=CHCOEt$, $Hg(OCOCF_3)_2$, reflux, 72h (g) pyrex helices, 250^oC, flow rate 0.16ml/s (h) $AgNO_3$, KOH, EtOH-H_2O, 0.5h (i) $SnCl_4$, CH_2Cl_2, -78^oC, 0.5H (j) pyrex helices, 185^oC (k) Ph_3CSCl, $CH_2=C(Me)OSiMe_3$, $ZnBr_2$ (cat), CH_2Cl_2, -78^oC, 1.5h (l) CH_2N_2, Et_2O-EtOH, rt, 8h (m) OsO_4 (cat), N-methyl morpholine N-oxide, t-BuOH, THF-H_2O, rt, 72h (n) 1,1-dimethoxycyclohexane, p-TsOH (cat), rt, 0.5h (o) $(MeO)_2POCH_2CO_2$-tBu, NaH, LiBr, THF, rt, 17h (p) Li(n-Bu)(i-Bu)$_2$AlH, THF, -78^oC, 1.5h (q) PCC, 4Å molecular sieves, CH_2Cl_2. final conversions are as detailed in ref 15.


+ (-)-1-Borneol
(80)
(81)
a,b,c,d
PhSe
OH
OR
(82)
(83)
e,f
OCH=CH2
(84)
(85)
g,h
OHC
(86)
(87)
i,j
R = BORN
(88)
(89)
ArSCl
ZnBr (k, l)
OSiMe
HO2C
(91)

Scheme 1.9

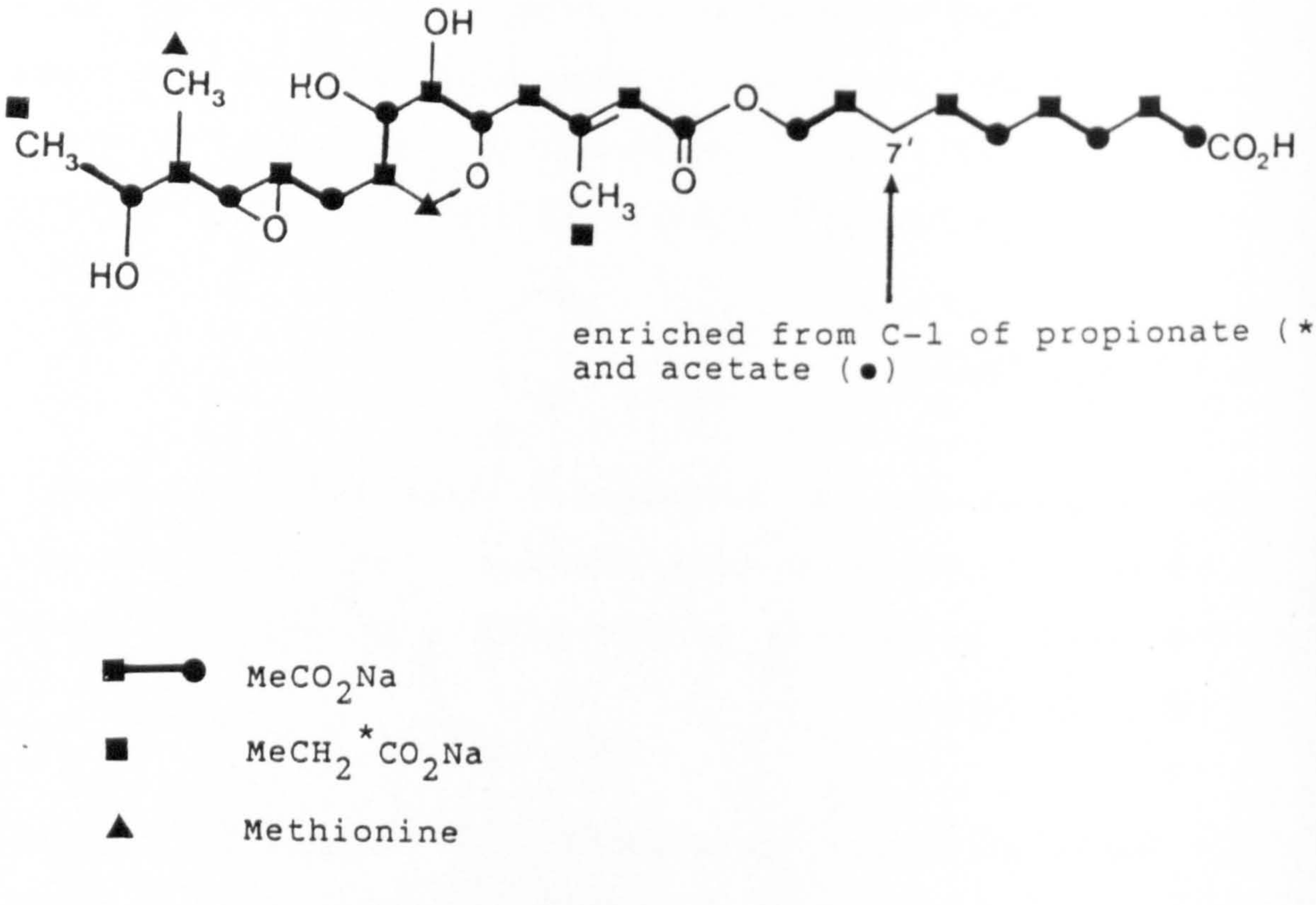
in the presence of 2-(trimethylsiloxy)propene was accompanied by elimination and lead to (88) and its trans isomer (89). Cis hydroxylation to (93), protection of the resulting diol (93) and Horner-Emmons condensation with tert-butyldimethylphosphono-acetate gave (94). Attachment of the second side chain of monic acid C was achieved by means of Julia olefination with a sulfone prepared from ethyl (2S,3S)-3hydroxy-2-methylbutanoate (95). The resulting tetrahydropyran after removal of the three protecting groups yieded (-)-monic acid C (79). This was then converted to pseudomonic acid C (5)[11a] and the latter, via its methyl ester was further elaborated to pseudomonic acid A (1)[12]. Hence synthesis of monic acid C thus constitutes a formal synthesis of these related materials.

1.3 BIOSYNTHESIS

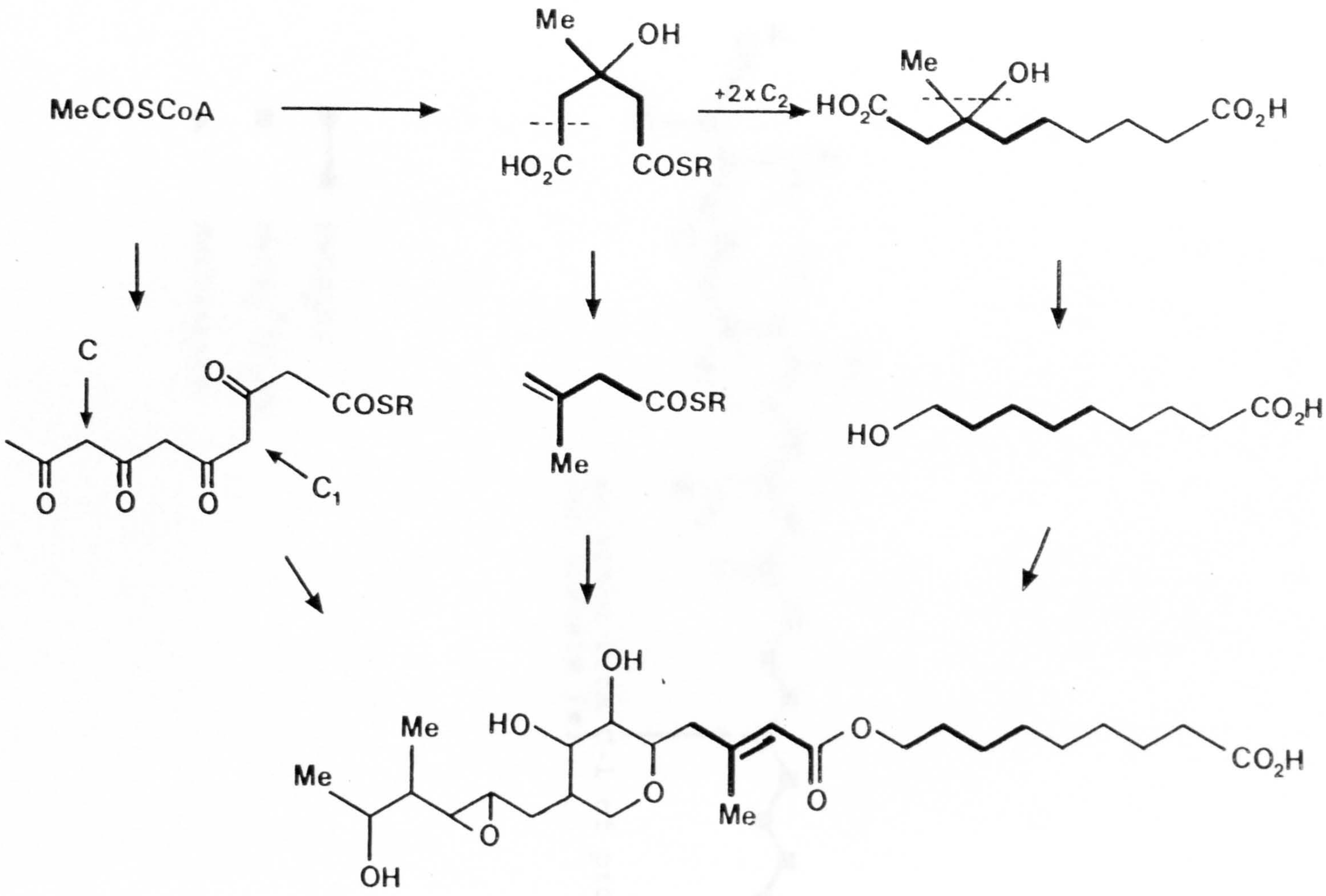
The biosynthesis of pseudomonic acid has also been studied[16]. These initial studies have indicated an essentially polyketide origin with a number of highly unusual features.

The basic biological precursors were defined by feeding a series of radio-isotopically labelled substrates as acetate, propionate and methionine. [2-^{14}C]Mevalonic acid lactone was not incorporated. This implied that pseudomonic acid does not contain an isoprenoid residue derivable from mevalonate.

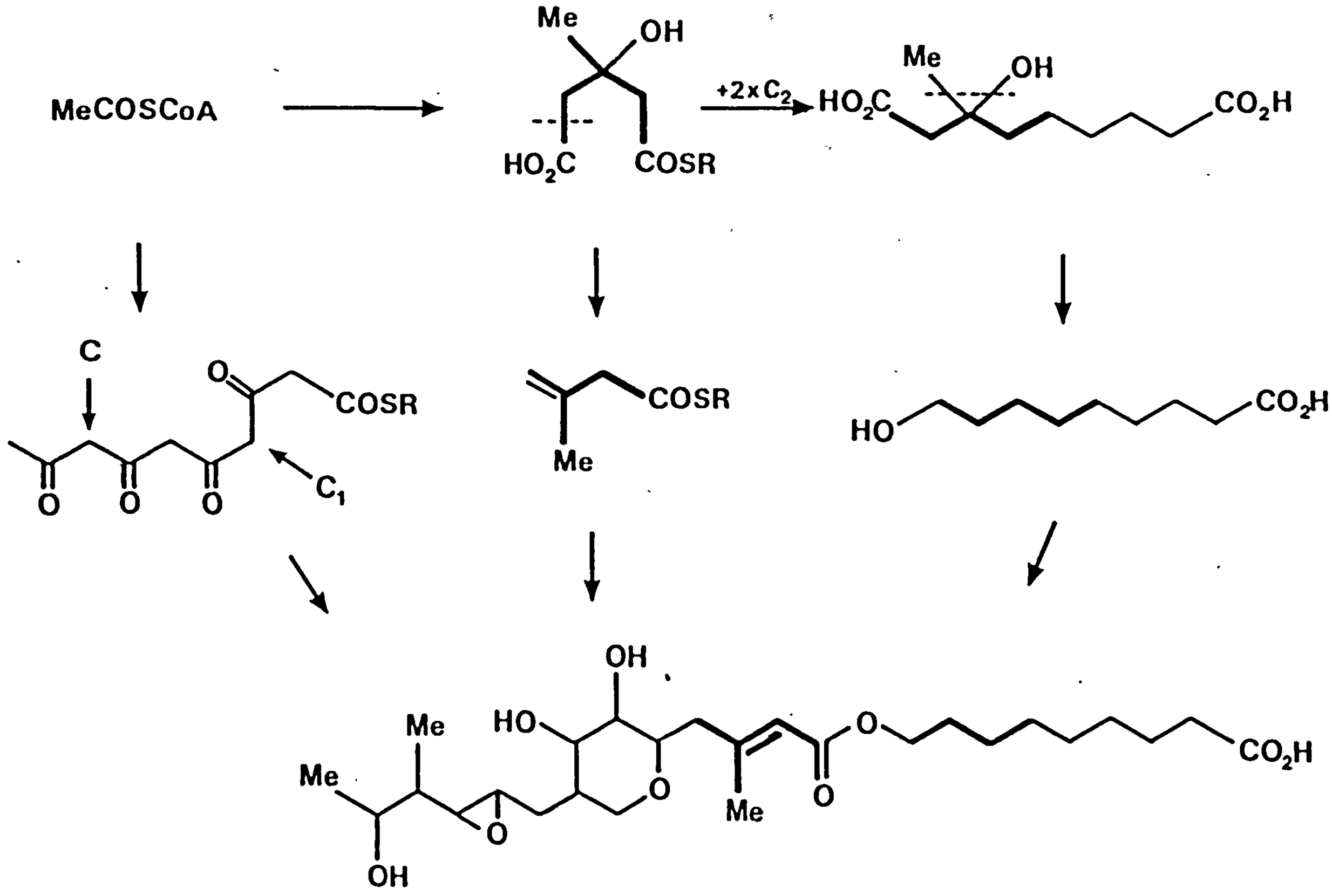
From these results many combinations of acetate, propionate


OH
HO
CH3
CH3
HO
O
O
O
O
CH3
7'
CO2H
enriched from C-1 of propionate (*)
and acetate (●)
MeCO2Na
MeCH2*CO2Na
Methionine

Scheme 1.10


MeCOSCoA
Me
OH
HO2C
COSR
+2xC2
HO2C
Me
OH
CO2H
C
O
COSR
C1
O
O
O
COSR
Me
HO
CO2H
OH
HO
Me
Me
O
O
O
Me
O
CO2H
OH

Scheme 1.11


MeCOSCoA
Me
OH
HO2C
COSR
+2xC2
HO2C
Me
OH
CO2H
C
O
COSR
C1
O
O
O
Me
HO
CO2H
OH
Me
Me
HO
O
O
O
Me
OH

Scheme 1.11

and methionine could be accommodated by the structure of pseudomonic acid. The C-17 fragment, monic acid, is composed of a continuous carbon chain extending from C-14 to C-1 with 'extra' C-1 units (carbons 15, 16 and 17) coming potentially from *S*-adenosylmethionine attached to carbons 3,8 and 12 respectively. Two chemical degradations of pseudomonic acid derived from D,L-[^{14}C]methionine, indicated that C-16 and C-17 were the most likely labelled sites for this precursor.

To help solve the problem of the difficult chemical degradations required to locate the sites of label from ^{14}C labelled precursors, ^{13}C labelled substrates were then used in conjunction with high field ^{13}C nmr spectroscopy to locate the labelled sites. The ^{13}C and ^{1}H nmr spectra of pseudomonic acid have now been rigorously assigned (see in section 2.3)[17]. However, for these initial studies the assignments used were achieved by comparison with spectra of the acetonide derivative, its C-13 ketone analogue and 8-methoxycarbonyl-octyl-3-methylbut-2-enoate. The resonances which could not be unambiguously assigned by this method, were distinguished from the incorporation of various ^{13}C acetates. Scheme 1.10 shows the labelling pattern deduced from these initial studies. The pathway proposed on the basis of these preliminary studies is summarised in scheme 1.11. According to this, pseudomonic acid is elaborated via C_{12}, C_5 and C_9 moieties.

1.3.1 The C_9 unit, 9-hydroxynonanoate moiety

This unit can be considered *a priori* to be derived

biosynthetically in a number of ways;

(a) from a preformed longer fatty acid, such as, oleic acid by oxidative degradation,

(b) from an even numbered carbon chain, such as, decanoic acid by α-oxidation, or

(c) from propionyl-CoA and malonyl-CoA by a process similiar to that leading to even numbered carbon chain fatty acids.

However, biosynthetic studies using ^{13}C labelled precursors have eliminated these alternatives since the observed sites of ^{13}C enrichment and the ^{13}C-^{13}C coupling pattern are not consistent with the expected sites. Since, C-1 and C-9′ are both derived from C-1 of acetate, the possibility of an enzyme-mediated Baeyer-Villiger type oxidation of a preformed acetate derived aliphatic chain can also be discounted. The incorporation of intact acetate units into C-8′ and C-9′; C-3′ and C-4′; C-1′ and C-2′ (and most probably C-5′ and C-6′) and the incorporation of only the carboxyl carbon of a separate acetate unit into C-7′ of the 9-hydroxynonanoate side chain suggests that the 'primer' for its formation may be β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) or a C_5 precursor derived from it. The labelling pattern of HMG-CoA from acetate is well known[18] since this substance is an important intermediate in the biosynthesis of mevalonate, the precursor of the "isoprene" unit in terpene biosynthesis. The addition of two malonyl-CoA residues to HMG-CoA or a C_5 'primer' derived from it are required if this is the biosynthetic route to this C_9 unit. The terminal carboxyl group would necessarily be reduced and

the β-methyl group cleaved.

This theory, does not account for the observed ^{13}C enrichment from [1-^{13}C]propionate solely into C-7′ and the reduced enrichment of this carbon from [1-^{13}C]- and [1,2-^{13}C]acetate.

To explain these observations, the existance of two competing pathways for the formation of this moiety was suggested, involving a second 'primer' from a different origin. Possibilities for this second 'primer' include homo-HMG-CoA or a C_5 unit derived from it (possibly the same C_5 unit derived from HMG-CoA). Some supporting evidence for homo-HMG-CoA (or the free acid) as an intermediate in metabolic pathways is the incorporation of homomevalonate, presumably derived from homo-HMG-CoA, into juvenile hormone[19] (96). However, Homo-HMG-CoA has never been isolated from natural sources.

These results may have important implications for the general biosynthesis of odd numbered carbon chain fatty acids which have been assumed to be synthesised from a propionyl-CoA 'primer'.

1.3.2 The C_{12} moiety

This unit comprises of a C_{10} chain extending from C-5 to C-14 of pseudomonic acid, to which two 'extra' carbon atoms, C-16 and C-17, have been attached at C-8 and C-12 respectively. The alternate labelling sequence from [1-^{13}C]- and [2-^{13}C]acetates along this chain and its formation from five intact acetate units accords with its

biosynthesis from an acetyl-CoA 'primer' and four malonyl-CoA units. In accord with the acetate-malonate hypothesis[20], the insertion of two 'extra' C_1 groups from *S*-adenosylmethionine would take place at saturated positions in the resulting polyketide chain. The functionalities present in this moiety, in the final metabolite, arise via oxidative and reductive modifications. Mellows *et al* proposed that the pyran ring was probably generated by eliminating water from an open-chain diol intermediate.

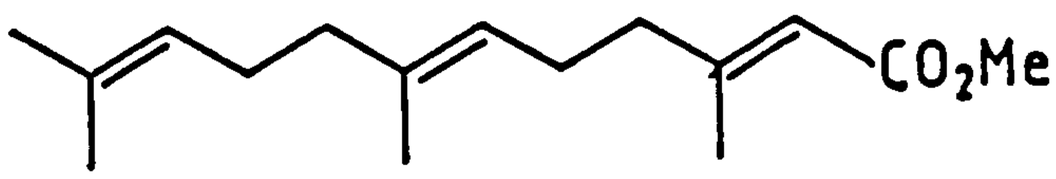
1.3.3 The C_5 moiety

Incorporation results from ^{13}C labelled acetates indicate this unit could be derived *via* HMG-CoA. It is known[21] that in rat liver HMG-CoA can undergo enzymic decarboxylation, producing hydroxyvaleryl-CoA, which can then undergo dehydration to produce dimethylacryloyl-CoA. The latter molecules have been shown to have the same labelling patterns from acetate as the C_5 moiety of methyl pseudomonate, ie.carbons 2,4 and 15 derived from C-2 of acetate; carbons 1 and 3 from C-1 of acetate and two intact acetate units forming carbons 1,2 and carbons 3,4. However, 3-methylbut-1-enyl CoA which could be formed by isomerisation of dimethylacryloyl-CoA may be a more appropiate condensing unit.

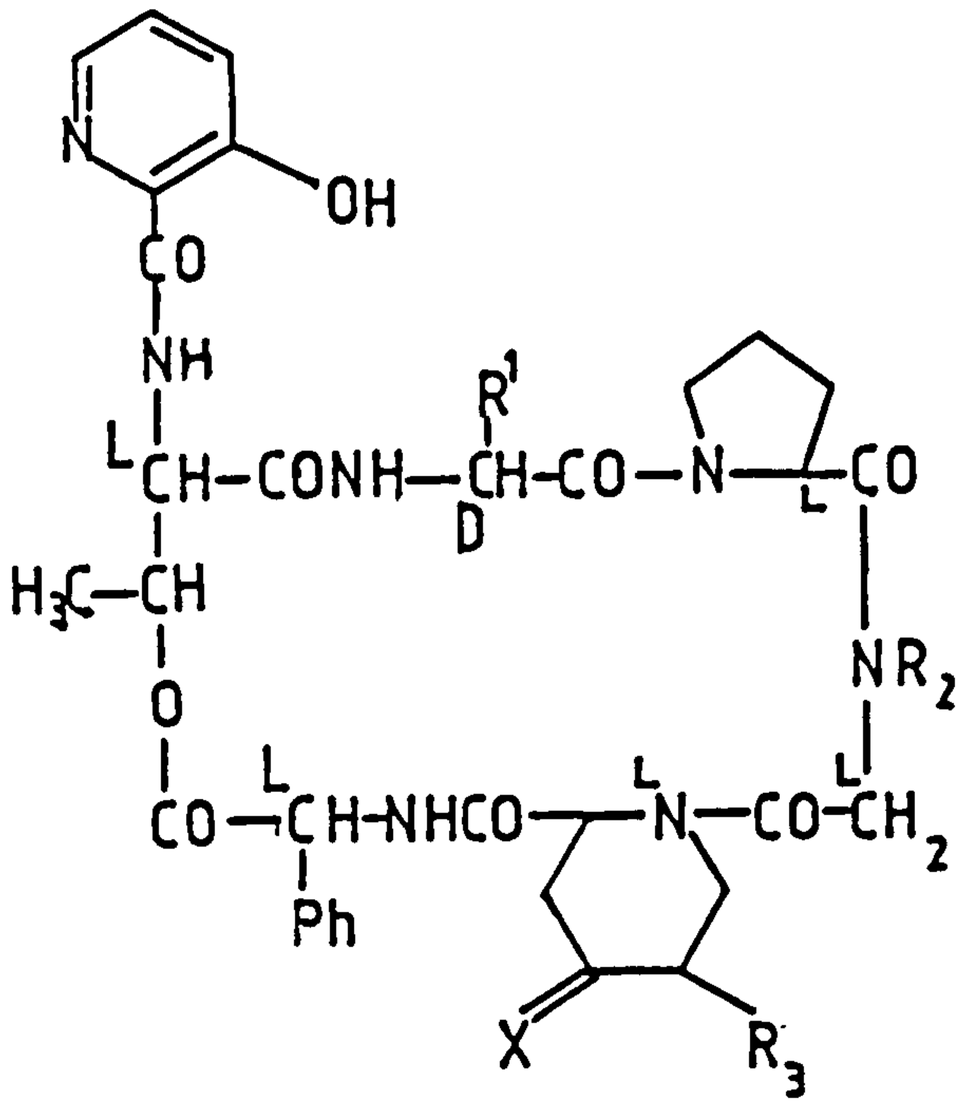
1.4 CONCLUSIONS

Amongst the unusual features of this proposed pathway are:

(i) The possible involvement of



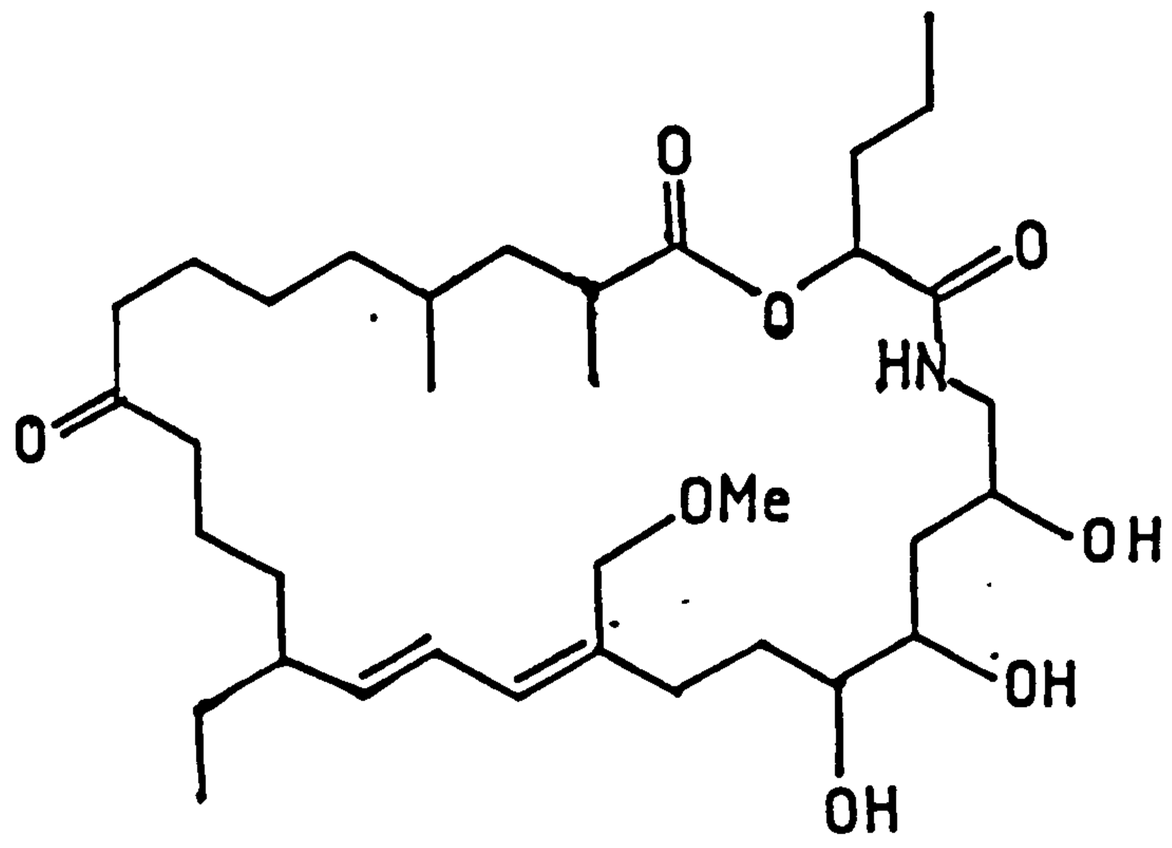
Insect juvenile hormone (96)



Virginiamycin S_1 (97) $R_1=R_2=CH_3$ $R_3=H$ X=O

Virginiamycin S_2 (98) $R_1=CH_2CH_3$ $R_2=R_3=H$ X=H, OH

Virginiamycin S_3 (99) $R_1=CH_2CH_3$ $R_2=CH_3$ $R_3=OH$ X=O



Myxovirescin (100)

β-hydroxy-β-methylglutarate in the formation of both the C_9 and the C_5 moieties,

(ii) the derivation of C-7′ from the carboxyl carbon of both acetate and propionate,

(iii) the origin of the C-15 methyl from the methyl of a cleaved acetate unit.

This feature has been observed more recently in the biosynthesis of the virginiamycins[22] (97, 98, and 99), myxovirescin[23] (100), the myxopyronines[24] (12 and 13) and the aurantinins[6] (10 and 11) but no satisfactory biosynthetic explanation has been established yet.

CHAPTER TWO

Biosynthesis of Pseudomonic Acid

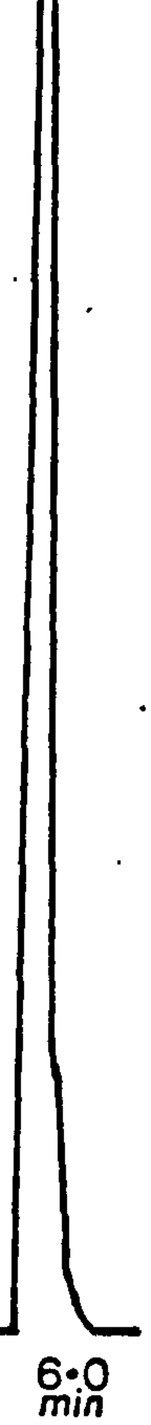
FIGURE 2.1
HPLC trace of a standard sample of pseudomonic acid obtained using a Waters μ-bondapak C-18 column, eluting at a flow rate of 1.5ml/min of methanol/0.05M ammonium acetate (3:2, v/v), pH4.5, uv detection at 230nm.

590μl Pseudomonic acid

0.2 OD 10μl sample

A = Pseudomonic Acid A

B = Pseudomonic acid B



2. INTRODUCTION

As discussed in chapter 1, *Pseudomonas fluorescens* (soil isolate, NCIB 10586), when grown in submerged culture produces the group of novel antibiotics collectively known as the pseudomonic acids. Extraction of the culture filtrates, followed by purification, provided materials containing over 90% pseudomonic acid A (1) and ca. 5% pseudomonic acid B (4) and minor amounts of pseudomonic acids C (5) and D (6). In the present work, two different fermentation media were investigated. Fermentation medium 1 was that used by previous workers[16] and fermentation medium 2 was that supplied by workers at Beecham[25], and was a modification of another published work[4]. Production of pseudomonic acid in these two media were compared. Analysis was by hplc. Figure 2.1 shows a typical trace for analysis of a standard sample of pseudomonic acid. Fermentation medium 2 produced the best yields and so was used in all subsequent experiments.

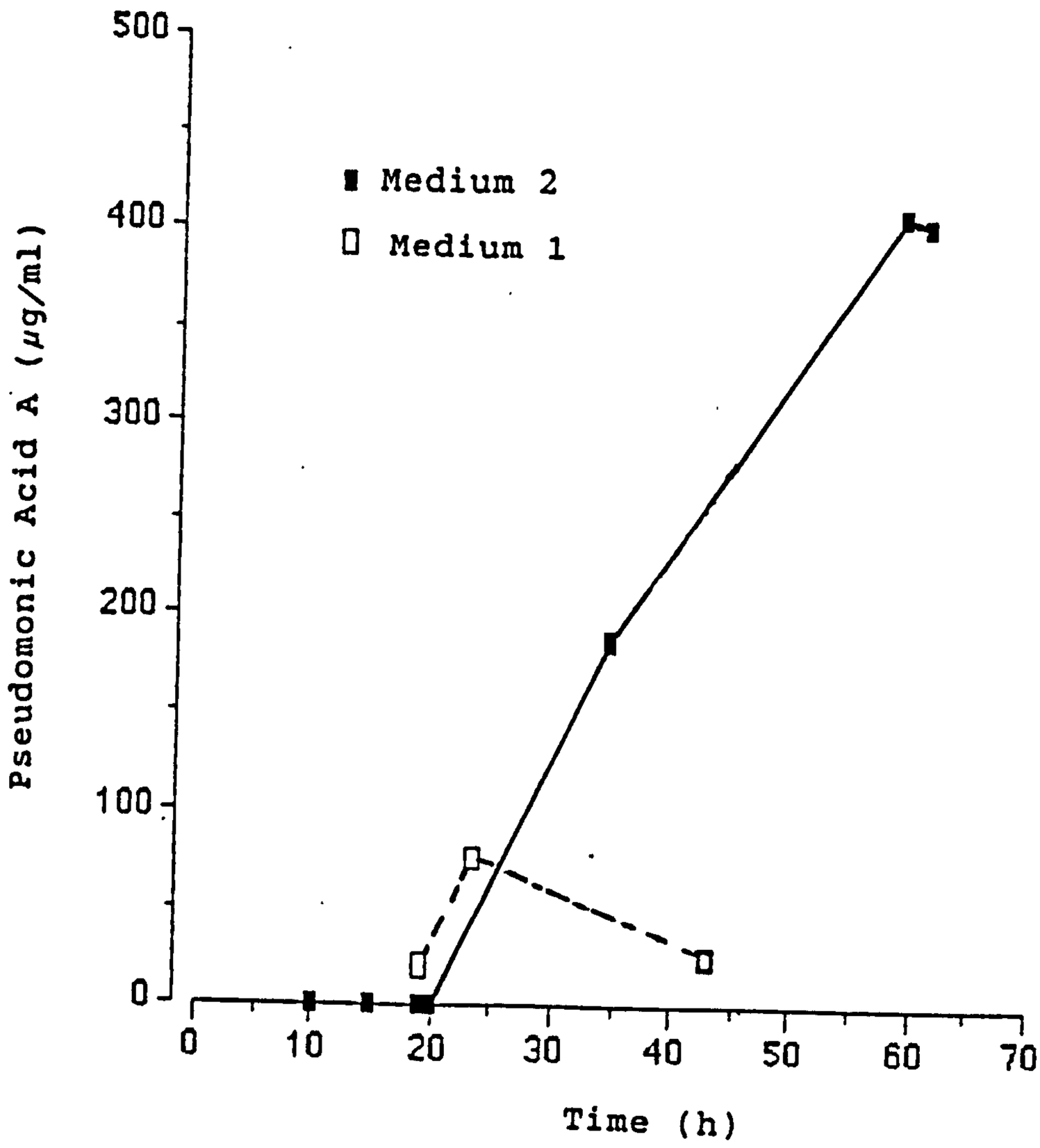
2.1 GROWTH PRODUCTION STUDY

For biosynthetic studies, it is necessary to determine the period of metabolite production, and this was achieved by carrying out a growth production study. Production of the pseudomonic acids involves a two stage fermentation. The bacterial culture is maintained on agar slopes at 4°C, and stage one involves transfering some bacterial cells from the

TABLE 2.1
Production of pseudomonic acid with time.

TIME AFTER INOCULATION (h)	μg/ml PSEUDOMONIC ACID A IN CULTURE FILTRATE
10	0
15	0
20	0
34.5	188
59	410
61	404

FIGURE 2.2
Growth production curve of pseudomonic acid with time.



storage slope into the primary stage liquid culture. This is used to obtain good bacterial cell growth. A sample of this liquid culture is then used to inoculate the second (production) stage liquid medium which contains all the required constituents for good production of the metabolites.

The preliminary growth production study used ten 250ml Erlenmeyer flasks, each containing 25ml of second stage medium. At timed intervals, 0.1ml of culture fluid was removed from all ten flasks, combined and after micro-filtration was analysed by hplc against a standard sample of pseudomonic acid of known concentration. The results are summarised in table 2.1 and figure 2.2. Several repetitions of this preliminary growth production study indicated that intial production of the metabolites occurred at approximately 20h and reached a maximum after 60h.

Another pre-requisite for ensuing biosynthetic studies was to determine the time for feeding labelled precursors to the second stage medium for maximum incorporation and good production of the metabolite. This information can be found from an incorporation experiment using [1-^{14}C]acetate and the specific activity of the pseudomonic acid isolated was assessed by liquid scintillation counting. The results, which are summarised in table 2.2, indicated that for good production and incorporation of label, labelled substrates should be added to the second stage medium after 20h growth. From earlier work[16], there are twelve labelled sites from acetate, hence the dilution per labelled site is equal

TABLE 2.2

Specific activities and dilutions per labelled site for methyl pseudomonate after incorporation of [1-^{14}C]acetate.

FERMENTATION TIME (h)	YIELD (mg)	SPECIFIC ACTIVITY (dpm/mmol)	DILUTION PER LABELLED SITE
10	30	$6.44X10^4$	848
15	20	$2.40x10^5$	22.7
20	15	$1.94x10^6$	0.28
CONTROL	50	———	———

to the specific activity times the number of labelled sites, giving the dilution values quoted in table 2.2. This meant that ^{13}C enrichment of about 10.8% could be expected if 90% enriched ^{13}C labelled acetates were fed to the culture under these experimental conditions. Again, repetition of this experiment verified these findings.

2.2 USE OF NMR IN BIOSYNTHETIC STUDIES

Apart from intial incorporation experiments, where radio-labelled precursors were used, substrates labelled with stable isotopes, ^{13}C, ^{2}H and ^{18}O were used in further studies. The sites of labelling were determined by nmr methods. The use of stable isotopes together with high field nmr has solved the problem of location of labelled sites in isolated metabolites. The use of radio-isotopes necessitates extensive degradative chemistry to locate the position of incorporated isotopic label. This degradative chemistry usually requires experimentally demanding and often impractical chemistry, especially where the metabolite has been labelled from a simple precursor such as acetate or mevalonate and is labelled in many sites. However, if accurate quantitative data on incorporation of labelled atoms into a metabolite is required, but the precise location of the label is not important, the use of radio-isotopes easily serves this purpose. This is the method of choice for establishing the most efficient precursor feeding regime prior to ^{13}C labelling studies. The precursor efficiency can be assessed by calculating the dilution of added label. For ^{14}C, dilution[26] per labelled

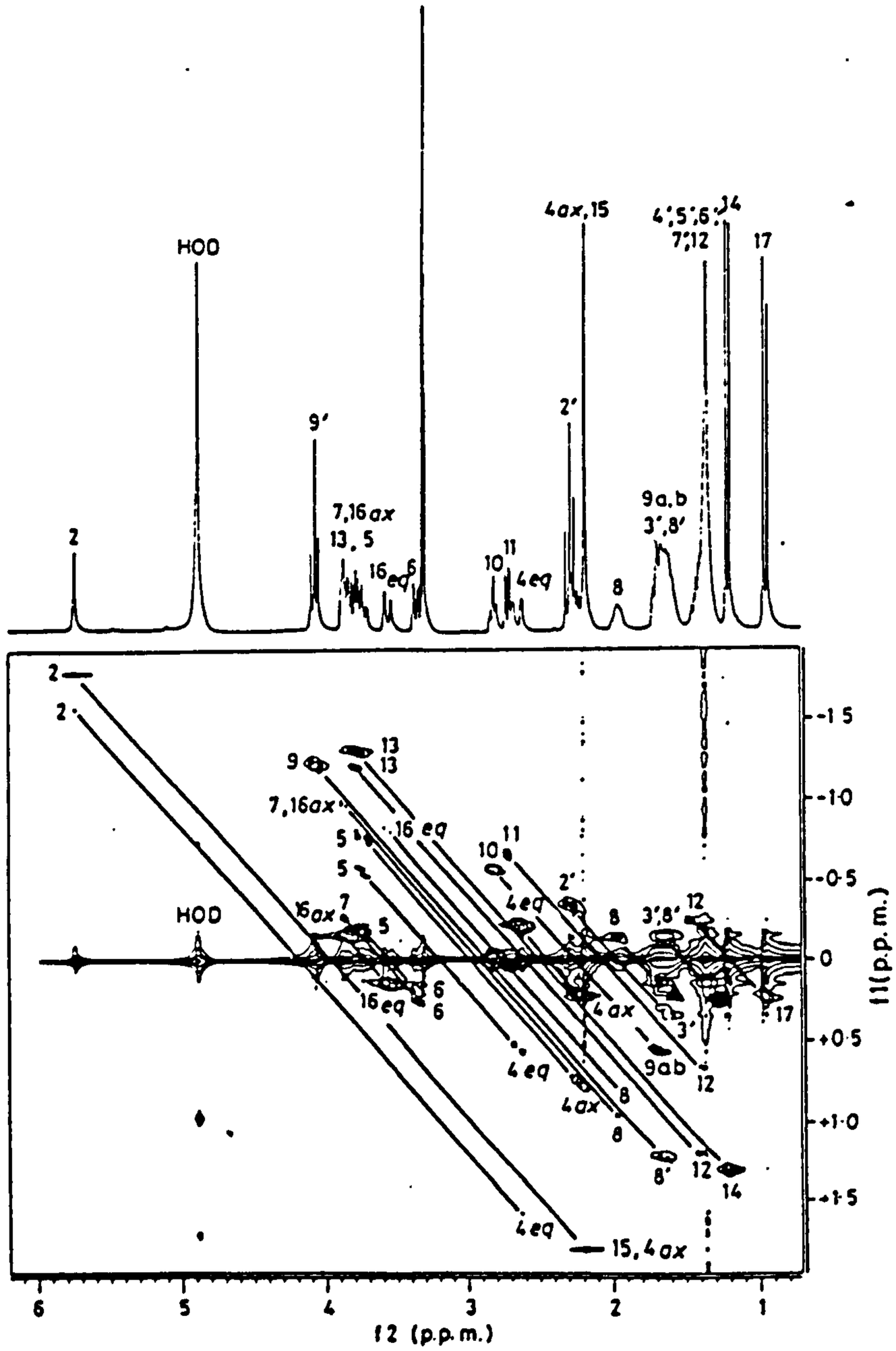
site is given by:

$$\frac{\text{specific activity of precursor}}{\text{specific activity of product}} \times \text{number of labelled sites}$$

The most important stable isotopes in biosynthetic studies are ^{13}C, ^{2}H, ^{18}O and ^{15}N. ^{13}C enables the biosynthetic origins of the carbon atoms in a molecule to be elucidated. The biosynthetic origins of the hydrogens and oxygens can be determined by use of ^{2}H and ^{18}O. With singly isotopically labelled substrates, mass spectrometry and ^{2}H nmr can be used to locate the sites of labelling. If ^{2}H or ^{18}O are used together with ^{13}C (used as a 'reporter' nucleus) then the labelled sites can be determined by observation of isotope induced shifts in the high field ^{13}C nmr of the isolated metabolite. The applications of stable isotopes and high field nmr to biosynthetic studies has been well reviewed by Simpson[26,27] and Vederas[28].

High field ^{13}C nmr provides a non-destructive detection system which can be used to determine the location and relative concentration of each chemically non-equivalent carbon in a molecule. It also, enables the presence of ^{2}H and ^{18}O to be demonstrated by use of isotope induced shifts in the ^{13}C resonance frequencies (or coupling) to ^{13}C. Since ^{18}O has a nuclear spin (I) equal to 0 it must be detected by indirect methods. However, ^{2}H has I=1, and so it can be detected directly but with nuclei of I=1, quadrupolar relaxation is observed and so the signals are broad. But, rapid relaxation and lack of any nOe means that accurate integration of ^{2}H nmr spectra is possible so that

FIGURE 2.3
A contour plot of the 250 MHz 2D ^{1}H SECSY(45°,N-type) spectrum of pseudomonic acid A in CD_3OD. Connectivities between scalar-coupled protons are indicated by the bold diagonal lines linking the relevant cross-peaks. The cross-peak marked with a triangle (▲) is due to H-9a,9b and that marked with a circle (●) is due to H-4,H-7'. The conventional 1D ^{1}H nmr spectrum is shown above the 2D spectrum.



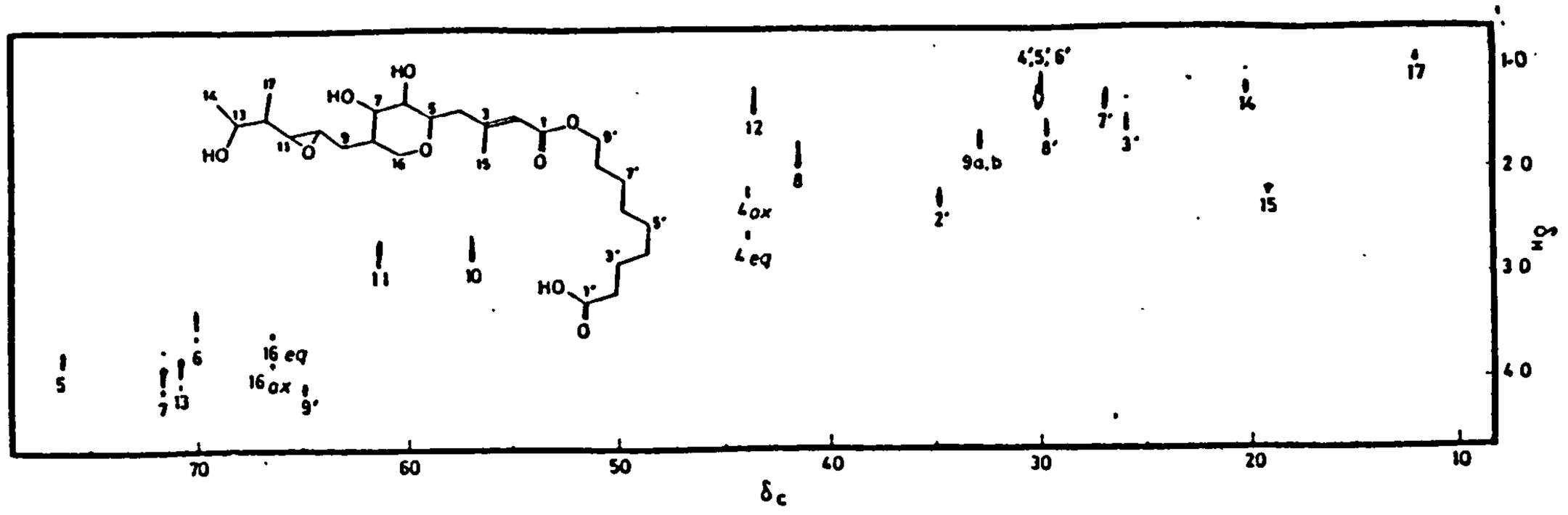
the relative enrichment at different sites in the metabolite can be assessed. Also, its low natural abundance (0.012%) means that much greater dilutions are tolerable, compared to ^{13}C (natural abundance 1.11%) which requires very efficient incorporation of the precursor into the metabolite. This makes ^{2}H-labelling attractive for the study of advanced intermediates in biosynthetic pathways. The inherent lack of resolution in ^{2}H nmr can be overcome by the use of isotope induced shifts in ^{13}C nmr. When deuterium is substituted either α or β to a ^{13}C, then this causes a change (usually upfield) in the ^{13}C chemical shift. Similarly, the presence of ^{18}O α to a ^{13}C atom can be detected by an upfield shift in the ^{13}C nmr spectrum.

2.3 ^{13}C AND ^{1}H NMR ASSIGNMENT OF PSEUDOMONIC ACID

The complete assignment of ^{1}H and ^{13}C nmr of a natural product is usually a pre-requisite for ensuing biosynthetic experiments in which stable isotopes are used. In the case of pseudomonic acid A, complete ^{1}H nmr spectral assignments were determined by Everett and Tyler[17] using a two dimensional (2D) chemical shift-correlated SECSY ^{1}H nmr. Figure 2.3 shows a contour plot of a (SECSY)^{1}H nmr spectrum of pseudomonic acid in CD_3OD.

In a SECSY spectrum, connectivities between scalar-coupled protons are detected by the presence of symmetrical cross-peaks in the f1 dimension of the 2D spectrum. These cross-peaks occur at half the chemical shift distance between coupled protons and they serve to identify mutually

FIGURE 2.4
A contour plot of the 2D $^{1}H,^{13}C$ correlation spectrum of pseudomonic acid A in CD_3OD.



adjacent protons in the structure. Starting from the olefinic proton H-2, which may be unambiguously assigned to the signal at δ5.73, connectivities can be seen to both 4-protons and the C-15 methyl. Further connectivities may then be traced in an unbroken sequence all the way across the ring and down the epoxide side chain to the C-14 methyl. In the nonanoic side chain, connectivities are seen from C-9′ to C-8′ to C-7′, and from C-2′ to C-3′ to C-4′. Thus the only protons whose chemical shifts cannot be unambiguously assigned are the C-5′ and C-6′ protons in the middle of this nonanoic acid side chain.

The assignment of the ^{13}C nmr[17] spectrum of pseudomonic acid A was achieved by a 2D ^{13}C, ^{1}H correlation spectrum, shown in figure 2.4. This type of 2D spectrum correlates the chemical shift of a proton with the chemical shift of the carbon to which it is directly bonded. For example, the chemical shift for the protons of the C-17 methyl occurs at δ0.94 in the ^{1}H nmr spectrum and in the 2D ^{1}H, ^{13}C correlation spectrum a cross-peak is seen at δ_H0.94, δ_C12.4. Hence, this unambiguously assigns the ^{13}C resonance of the C-17 methyl at δ_C12.4.

2.4 PRODUCTION OF THE PSEUDOMONIC ACIDS

In intial work, production of the metabolite was erratic. Reliable production was achieved for only a short time during which a number of experiments were carried out. Incorporation of [1-^{14}C]acetate, [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{2}H_3$]acetate, [3-^{14}C]-β-hydroxy-β-methylglutarate,

$^{16}O_2$ uptake measurements and finally $^{18}O_2$ experiments were carried out before yields of the metabolite became unacceptably low, and these experiments are discussed below.

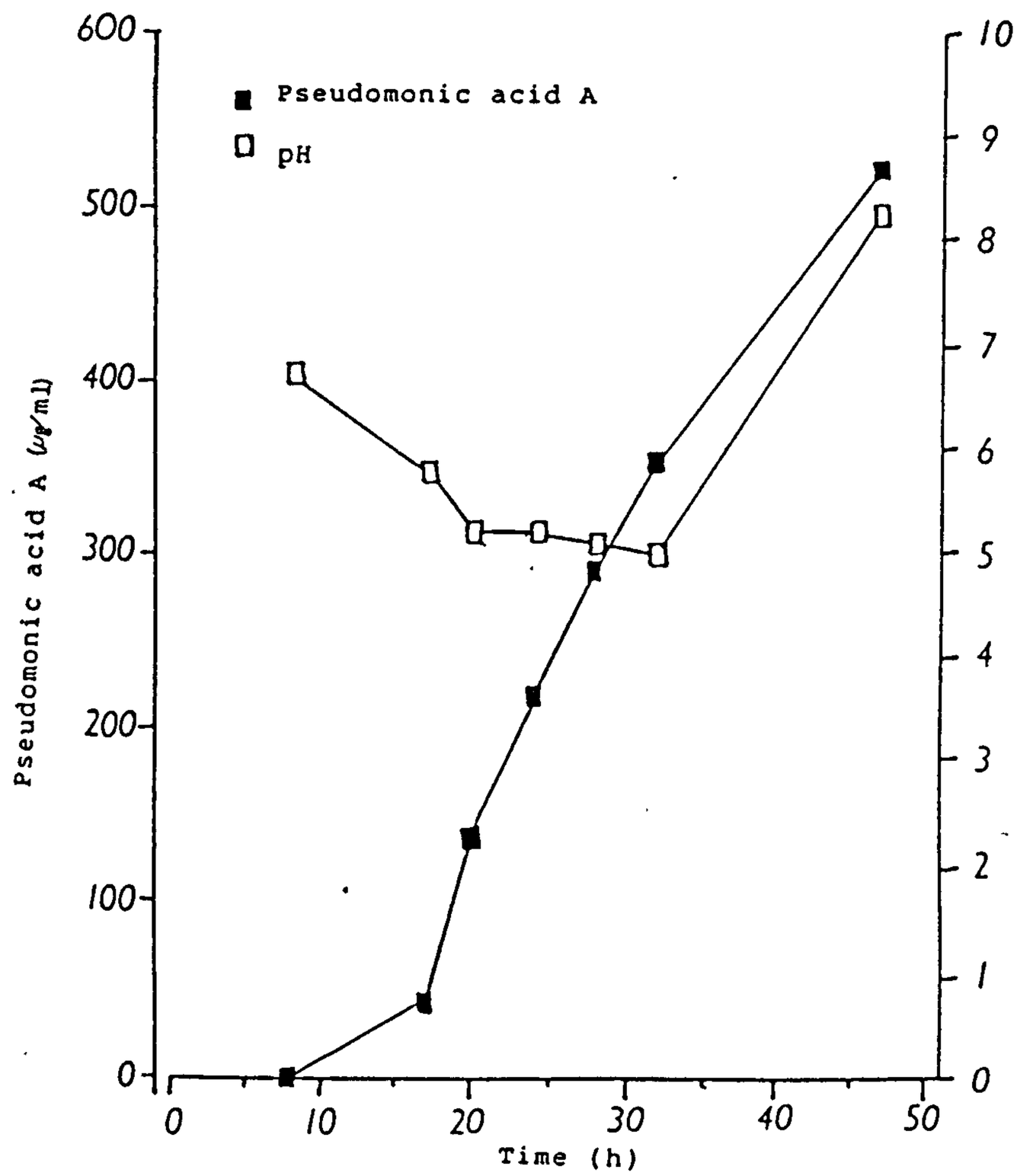
After consultation with workers at Beecham[25], the problem of poor production was traced back to subculturing of *P. fluorescens*. Intially the culture was maintained on nutrient agar slopes at +4°C and subcultured every 2 months. In subculturing, a loopful of bacteria from an original slope is transferred to a new slope which is then incubated at 25°C for 48h, so producing slopes with fresh cell growth. As with all living organisms, the original cells age and die and so by producing fresh cell growth the culture is maintained in a viable condition. However, on subculturing, vegetative growth of the bacterium remained unaffected but production of the metabolite decreased after each subculture. A set of working slopes - maintained on nutrient deficient agar slopes[25] - from a freeze-dried isolate were then set up and used to inoculate the second stage flasks. By using these nutrient deficient agar slopes, any active growth of the bacterium on the slopes is slowed down and so consequently the cells remain viable longer. Titres of the metabolites were also increased by using 'spiked' or 'baffled' flasks, in the second stage of the fermentation, which improve the degree of aeration of the medium as it is shaken on the rotary incubator by disrupting the circular motion of the liquid.

Another growth production study was carried out, again ten second stage flasks were used. This time 250ml 'baffled'

TABLE 2.3
Production of pseudomonic acid with time.

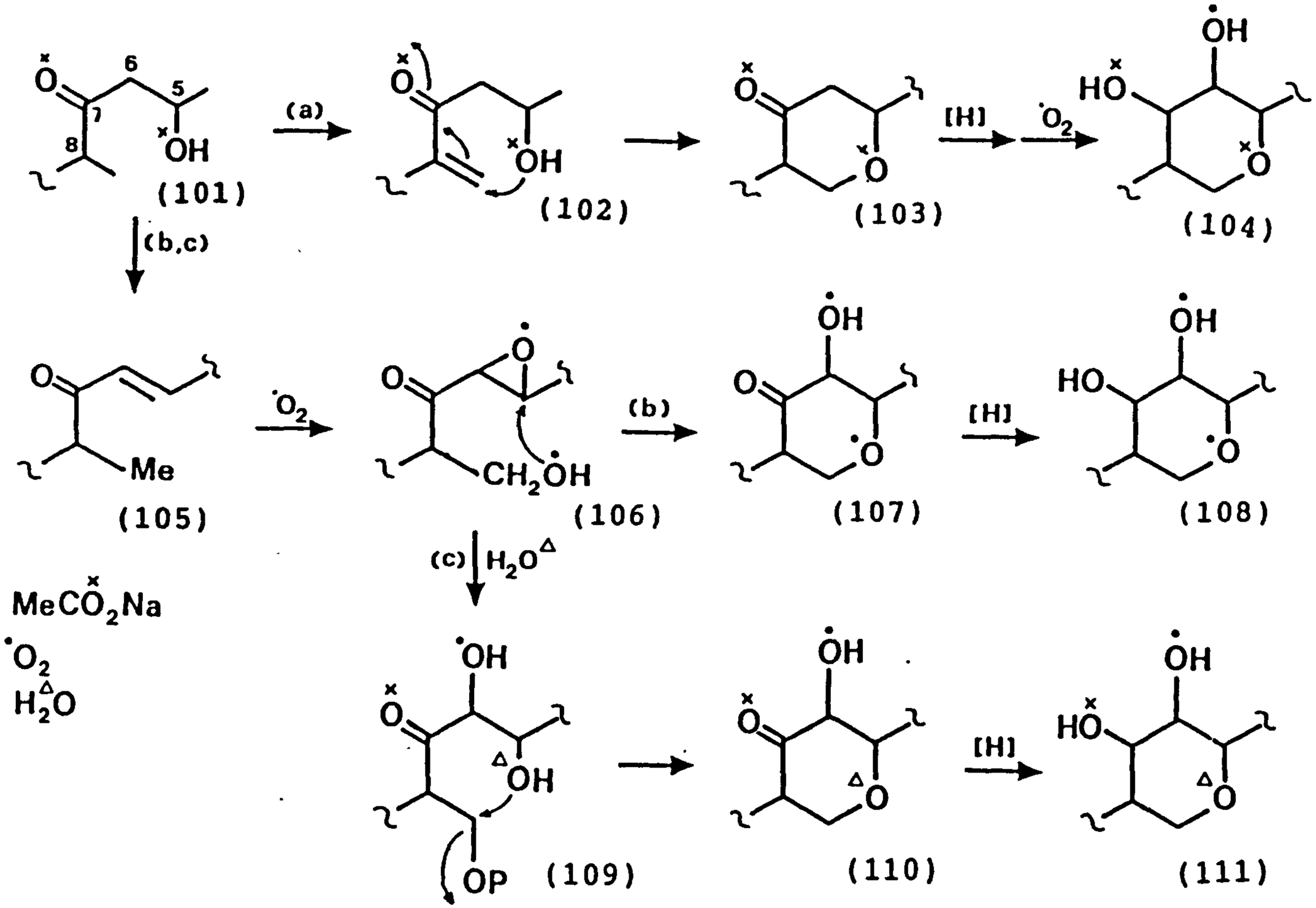
TIME AFTER INOCULATION (h)	pH	μg/ml PSEUDOMONIC ACID A IN CULTURE FILTRATE
8	6.7	0
17	5.8	45
20	5.2	139
24	5.2	220
28	5.1	290
32	5.0	354
47	8.3	523

FIGURE 2.5
Growth production curve for pseudomonic acid A.



flasks were used, 0.1ml was removed from each flask and combined, but this time the pH of this extract was adjusted to 8 and the cells removed by centrifugation. The pH change is required because at low pH pseudomonic acid is bound to protein and so the intial production is not detected if the pH is unadjusted. During the fermentation, the pH drops to approximately 4.5 as the glucose is used up by the bacteria and then the pH rises as the fermentation continues to end at approximately 8.0. The results of this study are shown in figure 2.5 and table 2.3. Incorporation studies which investigated the role of; [3,6-$^{13}C_2$]β-hydroxy-β-methyl-glutarate, malonate, bicarbonate and advanced precursor studies and the related 'check' feeds (chapter 3) were all performed using these modified culture and fermentation conditions.

RESULTS and DISCUSSION


(a)
(b,c)
[H]
O_2
(101)
(102)
(103)
(104)
(105)
(106)
(107)
(108)
(b)
(c) H_2O^{Δ}
$MeCO_2Na$
O_2
H_2O^{Δ}
(109)
(110)
(111)

Scheme 2.1

Results and Discussion

The incorporation of a variety of precursors labelled with stable isotopes has been studied in order to provide more detailed information on the exact nature of the intermediates involved in the formation of pseudomonic acid.

2.5 INCORPORATIONS OF [1-^{13}C,$^{18}O_2$]ACETATE, FERMENTATION IN AN ATMOSPHERE OF $^{18}O_2$, AND [1-^{13}C,$^{2}H_3$]ACETATES

There are a number of biogenetically reasonable mechanisms which can be proposed for the formation of the tetrahydropyran moiety depending on the structure of the intermediate produced by the chain assembly enzymes. Some of these are summarised in scheme 2.1, and they can be distinguished by determination of the biosynthetic origins of the hydrogen and and oxygen atoms.

In mechanism (a) the first step is dehydrogenation to generate an αβ-unsaturaterd ketone (102) which is then attacked by the hydroxyl group on C-5 in a 'Michael' type cyclisation to generate the intermediate pyran ring (103). This pyran ring is further modified by reduction of the ketone group on C-7 to a hydroxyl group and oxidation at C-6 to introduce another hydroxyl group at this position. A second possibility is illustrated by route (b). The first step is dehydration between C-5 and C-6 to generate another αβ-unsaturated ketone (106), which is then epoxised. The

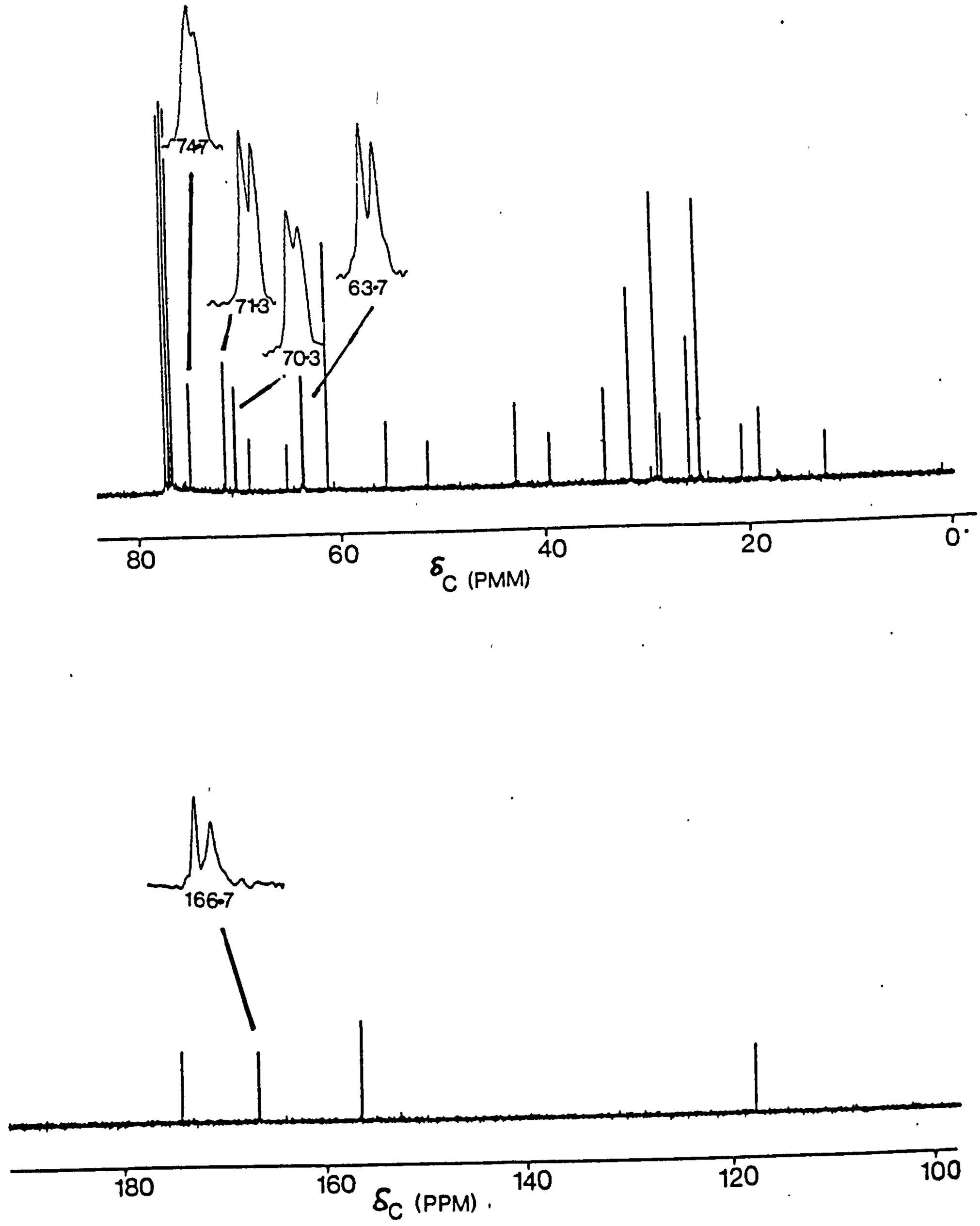
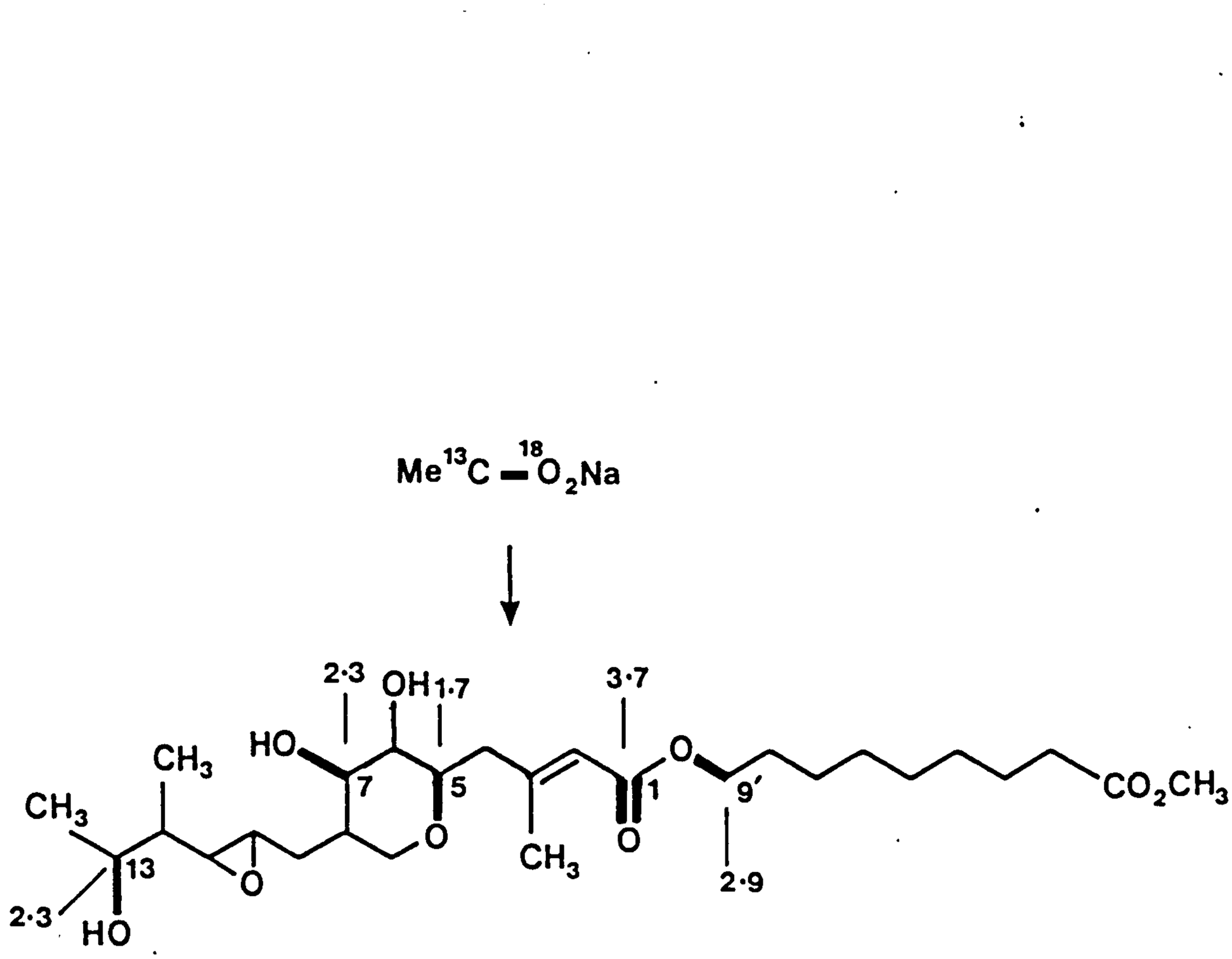


Figure 2.6 ^{13}C nmr spectrum of methyl pseudomonate labelled by [1-^{13}C,$^{18}O_2$]acetate.


Me13C═18O2Na
2·3
OH
1·7
3·7
HO
CH3
CH3
7
5
1
9′
CO2CH3
O
O
O
O
CH3
13
2·3
HO
2·9

Scheme 2.2

TABLE 2.4

^{2}H and ^{18}O isotope-induced shifts observed in the 90.56 MHz nmr spectrum of pseudomonic acid.

CARBON	δ_C (ppm)	$\Delta\delta$x100 (ppm)[a]	$\Delta\delta$x100 (ppm)[b]
1	166.7	3.7	
3	156.5		5.1
5	74.7	1.7	5.5
13	71.3	2.3	4.9, 9.1, 13.7
7	70.3	2.3	
9′	63.7	2.9	2.9
11	61.2		7.2
9	31.5		13.7
5′	29.0		10.1
3′	24.8		9.8

a [1-^{13}C,$^{18}O_2$]acetate enriched
b [1-^{13}C,$^{2}H_3$]acetate enriched.

methyl group on C-8 is hydroxylated and this introduced hydroxyl group attacks the epoxide to generate the pyran ring (107). The ketone on C-7 is reduced to a hydroxyl group, so producing the required tetrahydropyran ring with the substitution pattern found in pseudomonic acid. A third possibility, which is a variation on the second mechanism, is shown as route (c). Again the first step is dehydration followed by epoxidation to generate the intermediate (106). This time, the epoxide is hydrolysed to form the diol (109) which is cyclised via an activated phosphate to generate a pyran ring. The final modification is again reduction of the ketone group on C-7 to a hydroxyl group. This open-chain diol (109) was proposed by the original workers as being the most probable biosynthetic intermediate to the pyran ring.

Incorporation of [1-^{13}C,$^{18}O_2$]acetate and fermentation in an $^{18}O_2$ enriched atmosphere will enable the origin of all the oxygen atoms to be elucidated by observation of isotope induced shifts in the ^{13}C nmr spectra of the enriched metabolites. Incorporation of [1-^{13}C,$^{2}H_3$]acetate and analysis by ^{13}C nmr will enable the biosynthetic origins of the hydrogen atoms to be determined.

Sodium [1-^{13}C,$^{18}O_2$]acetate was successfully incorporated into pseudomonic acid. The sites of labelling in the enriched metabolite were determined by observation of isotope induced shifts in the ^{13}C nmr spectrum. The isotopic substitution with ^{18}O induces an upfield shift in the position of the nmr resonances of a directly attached ^{13}C atom, these are α (1 bond) shifts. The ^{13}C nmr spectrum

FIGURE 2.7

Apparatus for the growth of cultures in an $^{18}O_2$ atmosphere. The wash bottles are arranged so that the first acts as a suck-back trap, the second contains 5M KOH to absorb CO_2 produced by the cultures and the third contains cotton wool to remove any alkaline spray.

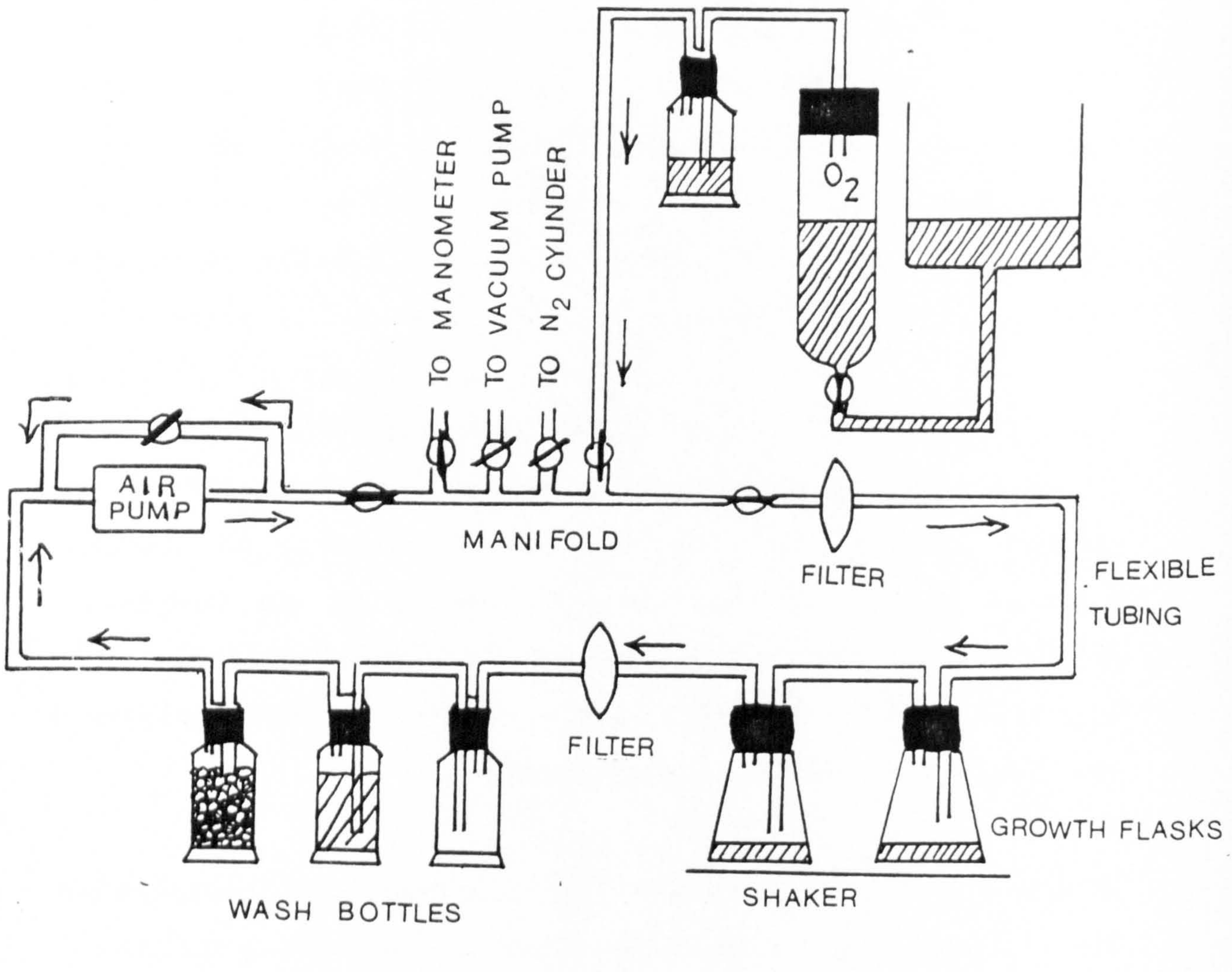
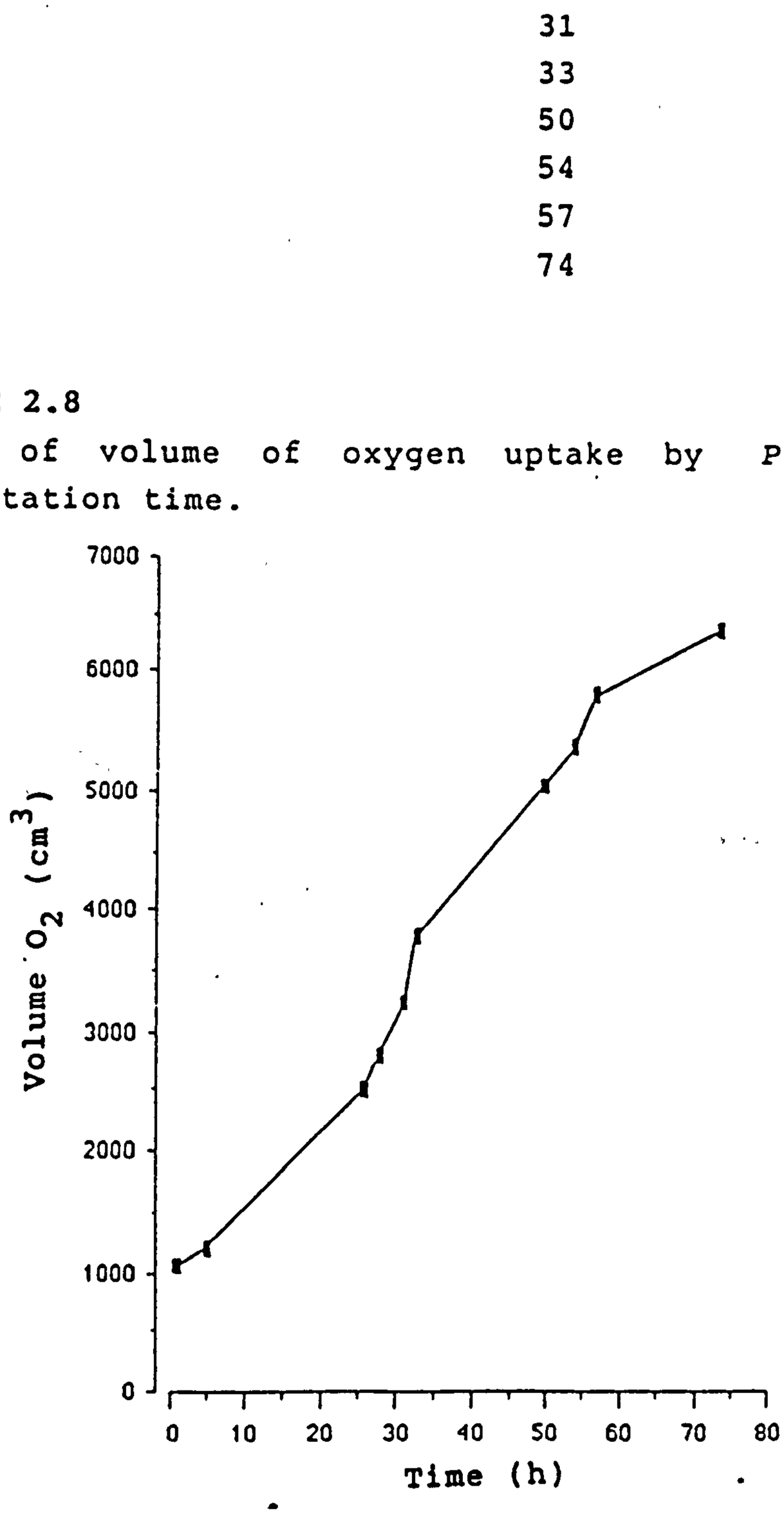


TABLE 2.5
Volume of oxygen uptake with fermentation time.

FERMENTATION TIME (h)	Vol. $^{16}O_2$ UPTAKE (cm^3)
1	1071
5	1197
26	2519
28	2796
31	3250
33	3779
50	5063
54	5403
57	5844
74	6386

FIGURE 2.8
Graph of volume of oxygen uptake by *P. fluorescens* versus fermentation time.



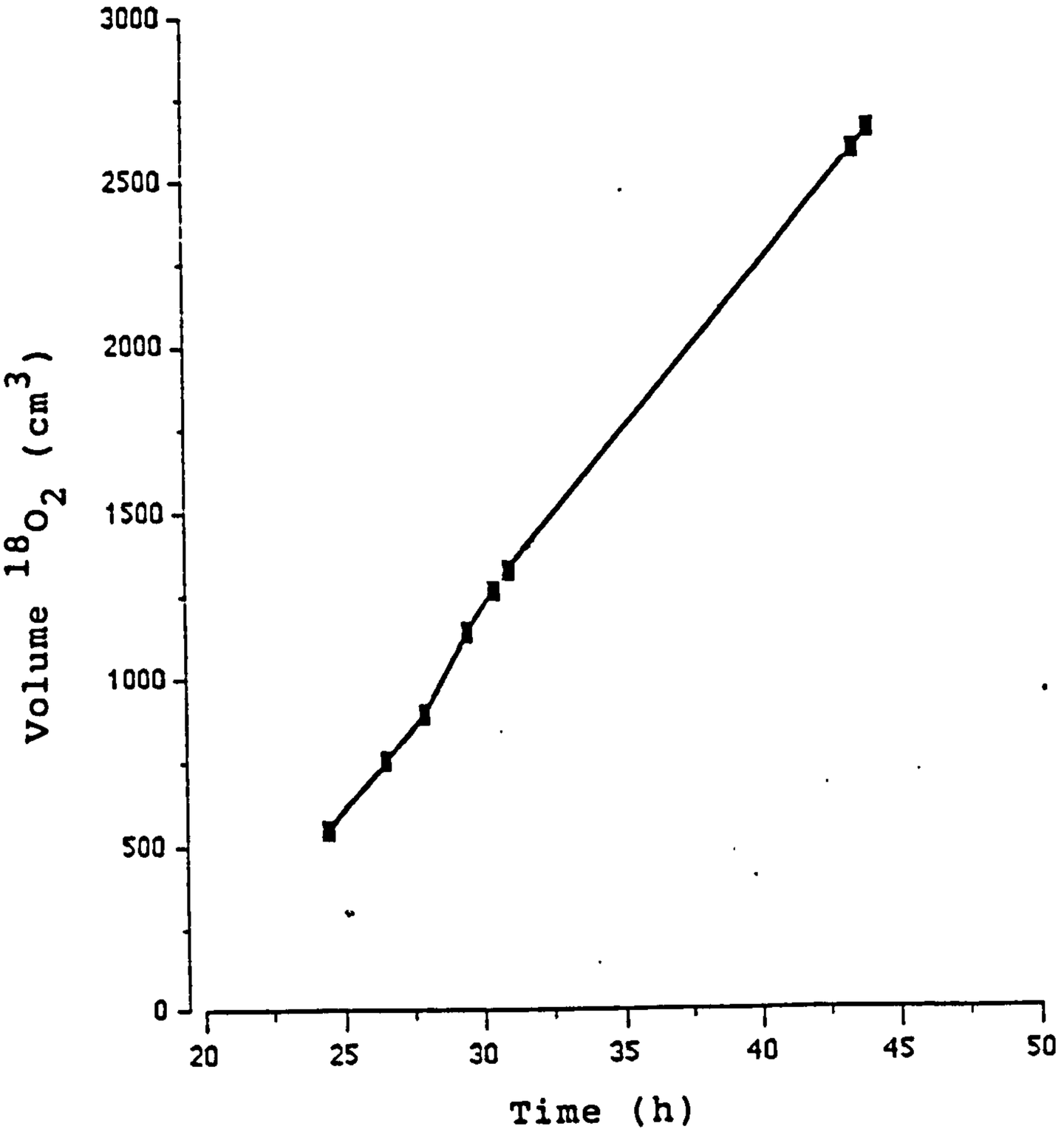
(figure 2.6) of this enriched metabolite showed five shifted signals and these are detailed in table 2.4. Hence, the oxygen atoms attached to C-1, C-5, C-7, C-13 and C-9′ (indicated in scheme 2.2) are all derived from acetate.

In an attempt to ascertain the origins of the remaining four oxygen atoms present in pseudomonic acid (*ie.* the epoxide, C-4 hydroxyl and the carboxylic acid oxygen atoms), *P. fluorescens* was grown in an atmosphere of $^{18}O_2$ gas, using the apparatus, shown in figure 2.7, which allows the oxygen pressure to be kept constant and the oxygen uptake to be monitored. Due to the expensive nature of $^{18}O_2$ gas, an intial experiment using $^{16}O_2$ was carried out to measure the oxygen uptake during fermentation of the culture. This also served to check that growing the culture in this unnatural atmosphere, *ie.* where the CO_2 produced is constantly removed from the system, had no adverse affect on the production of the metabolite. This is indeed the case. Figure 2.8 and table 2.5 relates oxygen uptake by the culture to fermentation time. These show that, the maximum uptake of oxygen occurs from 24h and has passed its maximum level by 45h. Hence, for the most economical use of $^{18}O_2$ gas, the culture was grown for 24h, which is the intial growth phase of the bacterium, before being attached to the closed ^{18}O apparatus for the period of metabolite production. In the intial $^{16}O_2$ uptake study, the fermentation was continued for 72h, the time required for maximum metabolite production. However, since the oxygen uptake by the culture has passed its maximum level by 45h, the fermentation in the closed system using $^{18}O_2$ gas was stopped after 44h fermentation.

TABLE 2.6
Volume of $^{18}O_2$ uptake with fermentation time.

FERMENTATION TIME (h)	VOL. $^{18}O_2$ UPTAKE (cm^3)
24.5	554
26.5	743
28.0	894
29.5	1146
30.5	1265
31.0	1328
43.5	2600
44.0	2650

FIGURE 2.9
$^{18}O_2$ utilization by *P. fluorescens* versus time.



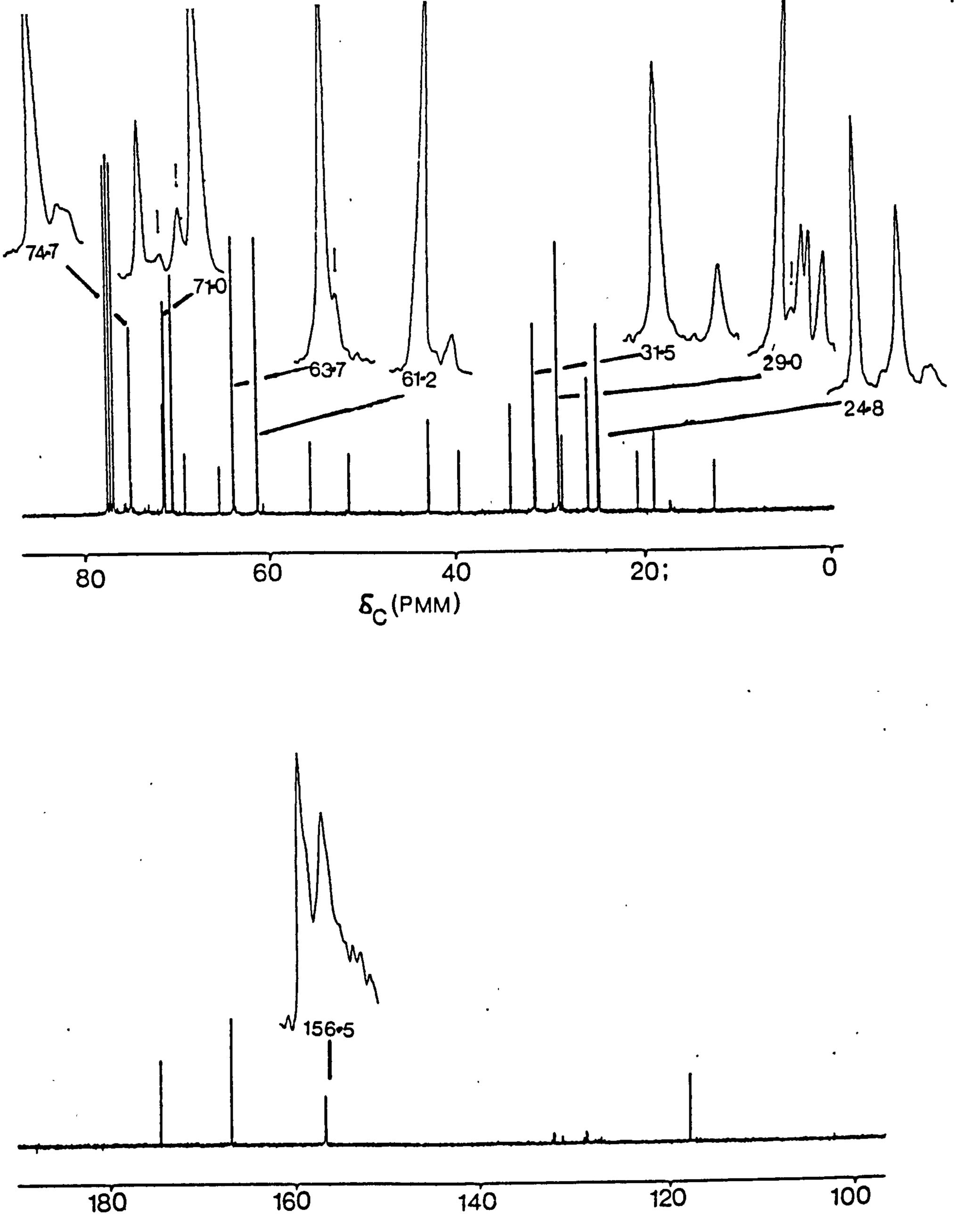
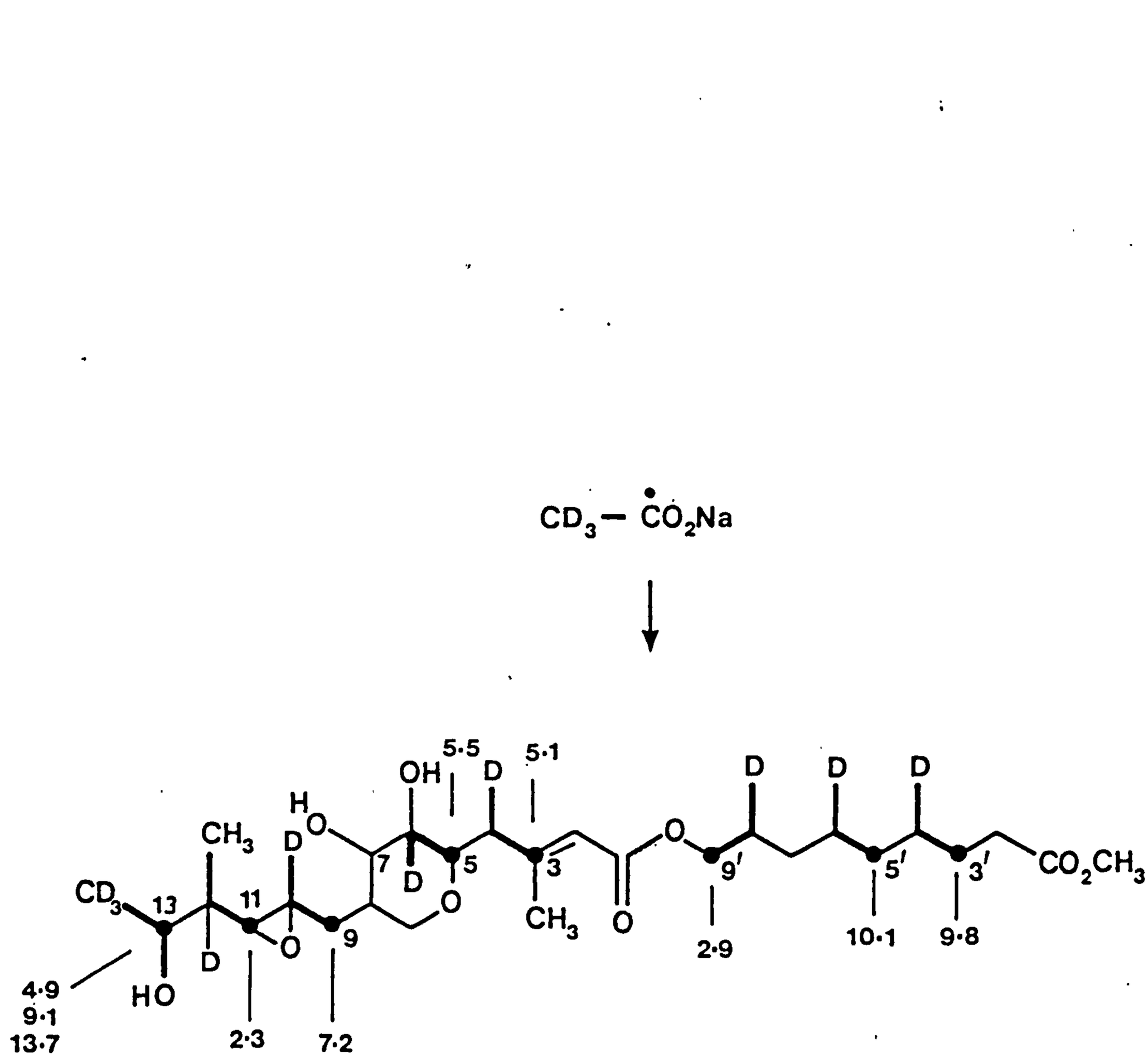


Figure 2.10 ^{13}C nmr spectrum of methyl pseudomonate labelled by [1-^{13}C,$^{2}H_3$]acetate.

Table 2.6 and figure 2.9 illustrate the $^{18}O_2$ utilization with fermentation time. However, in this experiment, production of the metabolite was poor and only 8mg of a white solid was isolated from this experiment. The ^{13}C nmr spectrum of this isolated material was obtained but the sample was so weak, that apart from proving that the sample was indeed methyl pseudomonate, observation of any α shifts induced by incorporation of ^{18}O was not possible. To try and determine if any ^{18}O had been incorporated, high resolution mass spectrometry was tried. Unfortunately, due to problems obtaining this analysis, no information about the origins of the remaining four oxygen atoms was elucidated.

Sodium [1-^{13}C,$^{2}H_3$]acetate was also sucessfully incorporated into pseudomonic acid, and the resulting enriched metabolite was analysed by high field ^{13}C nmr. A number of isotopically shifted signals, corresponding to β shifts (2 bond) were observed in this spectrum (figure 2.10, table 2.4). These upfield shifts in the positions of the ^{13}C resonances are due to the incorporation of ^{2}H on the carbon atom two bonds away from the carbon showing the shifted signal(s). There are three shifted signals at C-13, corresponding to the incorporation of up to three deuteriums on C-14 indicating that this is part of an acetate chain 'starter' unit. There are also singly shifted signals for the incorporation of a single ^{2}H, from acetate, on C-4, C-6, C-10, C-12, C-8', C-6' and C-4'. Interestingly, there is no observed shifted signal for C-7, indicating that no ^{2}H was incorporated on C-8. Scheme 2.3 shows the positions of the


CD3— ĊO2Na
CD3
13
CH3
D
11
HO
D
O
9
H
O
OH
7
D
5
5·5
D
5·1
3
CH3
O
O
9′
2·9
D
D
D
5′
3′
CO2CH3
10·1
9·8
4·9
9·1
13·7
2·3
7·2

Scheme 2.3

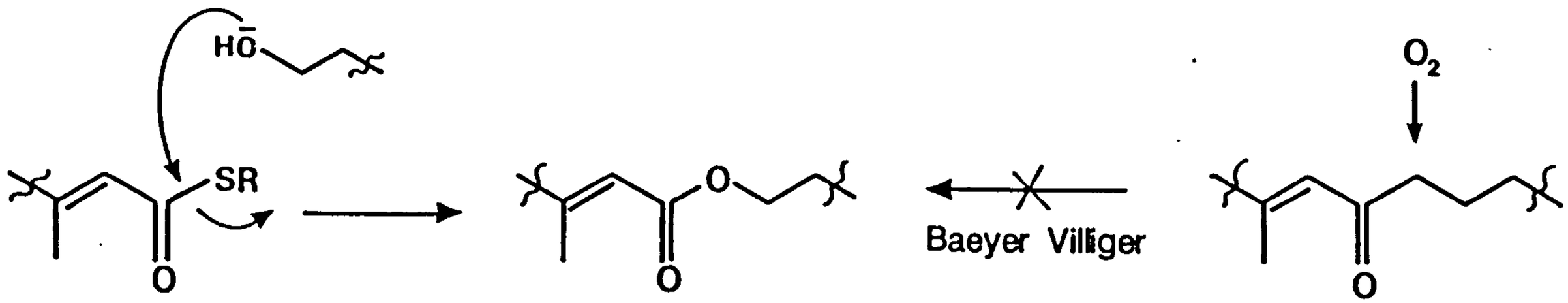
incorporated ^{2}H in the isolated enriched metabolite.

From the [1-^{13}C,$^{18}O_2$]acetate incorporation experiment, five oxygen atoms came from acetate, ie. the oxygen atoms at C-1, C-5, C-7, C-13 and C-9', and this result can be used to distinguish between the possible mechanisms for the formation of the tetrahydropyran ring.

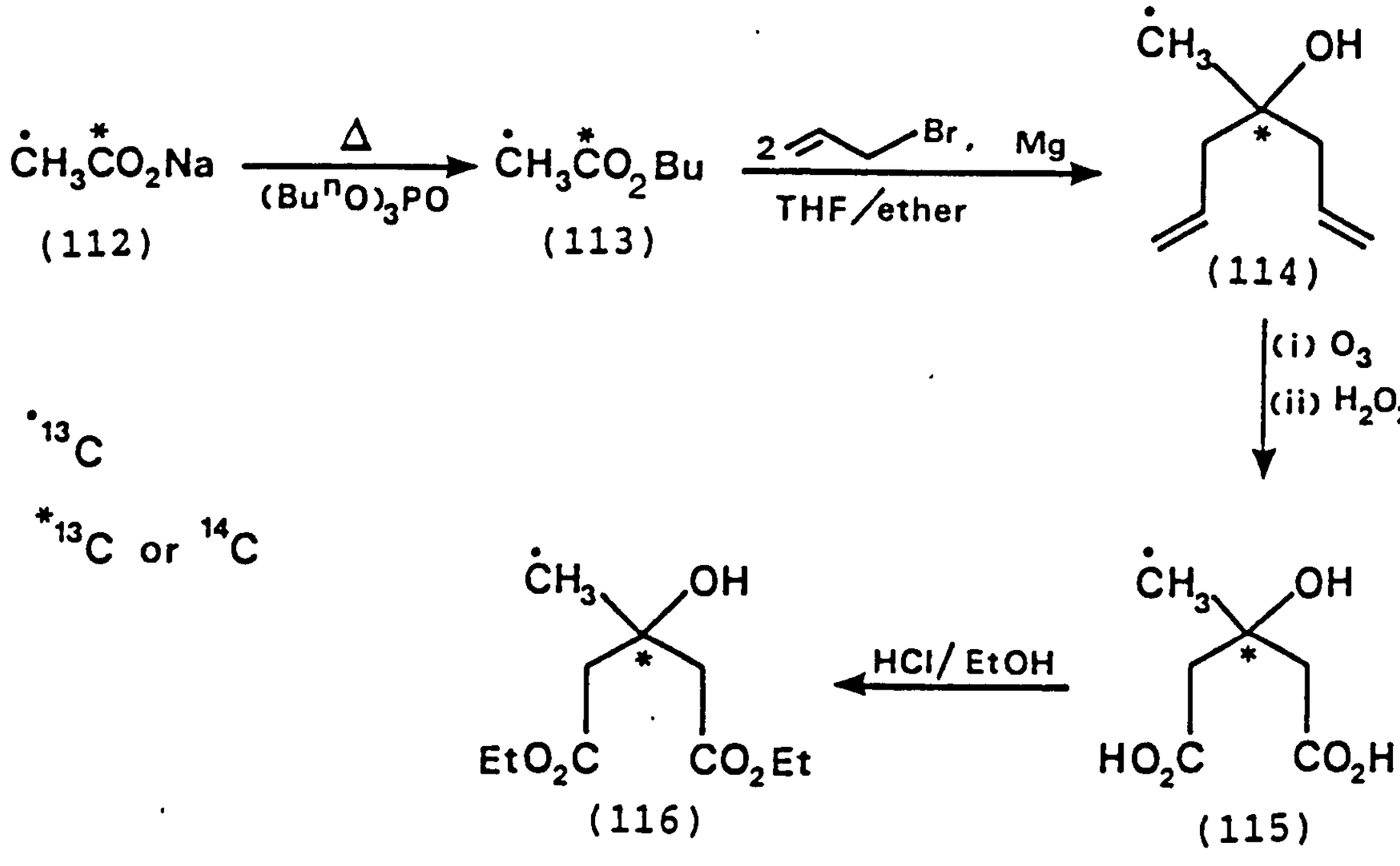
Therefore from the knowledge of the origins of the oxygen atoms, only mechanism (a) in scheme 2.1 is consistent with the observed labelling pattern. Routes (b) and (c), by first dehydrating between C-5 and C-6, lose the oxygen atom at C-5 which is acetate derived. This is actually maintained in the final metabolite, as shown by an α shift observed in the ^{13}C nmr spectrum at the C-5 resonance.

Indirect supporting evidence for route (a) being the mechanism for the formation of the pyran ring comes from the observed ^{2}H labelling pattern from [1-^{13}C,$^{2}H_3$]acetate. No β shift was observed at the C-7 resonance in the ^{13}C nmr spectrum of the enriched metabolite, and this indicates that the ^{2}H which comes from acetate has been lost in the biosynthetic transformations to pseudomonic acid. This is consistent with route (a) since the first step in this mechanism is dehydrogenation between C-8 and C-16 with the loss of ^{2}H from acetate. In all the other routes (b) and (c), the ^{2}H from acetate would be maintained in the final metabolite which is inconsistent with the observed labelling pattern.

The observed labelling pattern from [1-^{13}C,$^{18}O_2$]acetate



Scheme 2.4


$\dot{C}H_3\overset{*}{C}O_2Na$
(112)
Δ
$(Bu^nO)_3PO$
$\dot{C}H_3\overset{*}{C}O_2Bu$
(113)
2
Br, Mg
THF/ether
$\dot{C}H_3$
OH
*
(114)
(i) O_3
(ii) H_2O_2/H^+
$\dot{C}H_3$
OH
*
HO_2C
CO_2H
(115)
HCl/ EtOH
$\dot{C}H_3$
OH
*
EtO_2C
CO_2Et
(116)
$^{\bullet 13}C$
$^{*13}C$ or ^{14}C

Scheme 2.5

incorporation also establishes that both oxygen atoms involved in the unsaturated ester link between monic acid and 9-hydroxynonanoic acid are acetate derived. This confirms earlier findings by Mellows and co-workers[16] that pseudomonic acid is not derived *via*, for example, a Baeyer-Villiger type cleavage of a single long chain ketone intermediate but from separate C_{17} and C_9 moieties. Scheme 2.4 illustrates this point.

2.6 SYNTHESIS AND INCORPORATION OF [3-^{14}C]- AND [3,6-$^{13}C_2$]-β-HYDROXY-β-METHYLGLUTARATE

Intial biosynthetic studies by Mellows and co-workers[16] implicated β-hydroxy-β-methylglutarate in the biosynthetic pathway to pseudomonic acid (scheme 1.11). This proposed involvement of β-hydroxy-β-methyl-glutarate (HMG) in the formation of the C_9 and C_5/C_{17} moieties by providing C-3, C-4 and C-7′ of pseudomonic acid has now been tested by its synthesis in ^{14}C and doubly ^{13}C labelled forms and subsequent feeding studies.

The synthetic route to this precursor has been modified from a previous synthesis of mevalonic acid[29]. Due to the expensive nature of ^{14}C and ^{13}C labelled starting materials, all syntheses were first carried out using unlabelled starting materials in order to optimise reaction conditions. Scheme 2.5 details this synthesis. Step 1, involves the conversion of labelled sodium acetate (112) into labelled ^{n}butyl acetate (113) in a transesterification reaction with tri-^{n}butyl phosphate[31]. The ^{n}butyl acetate was then reacted

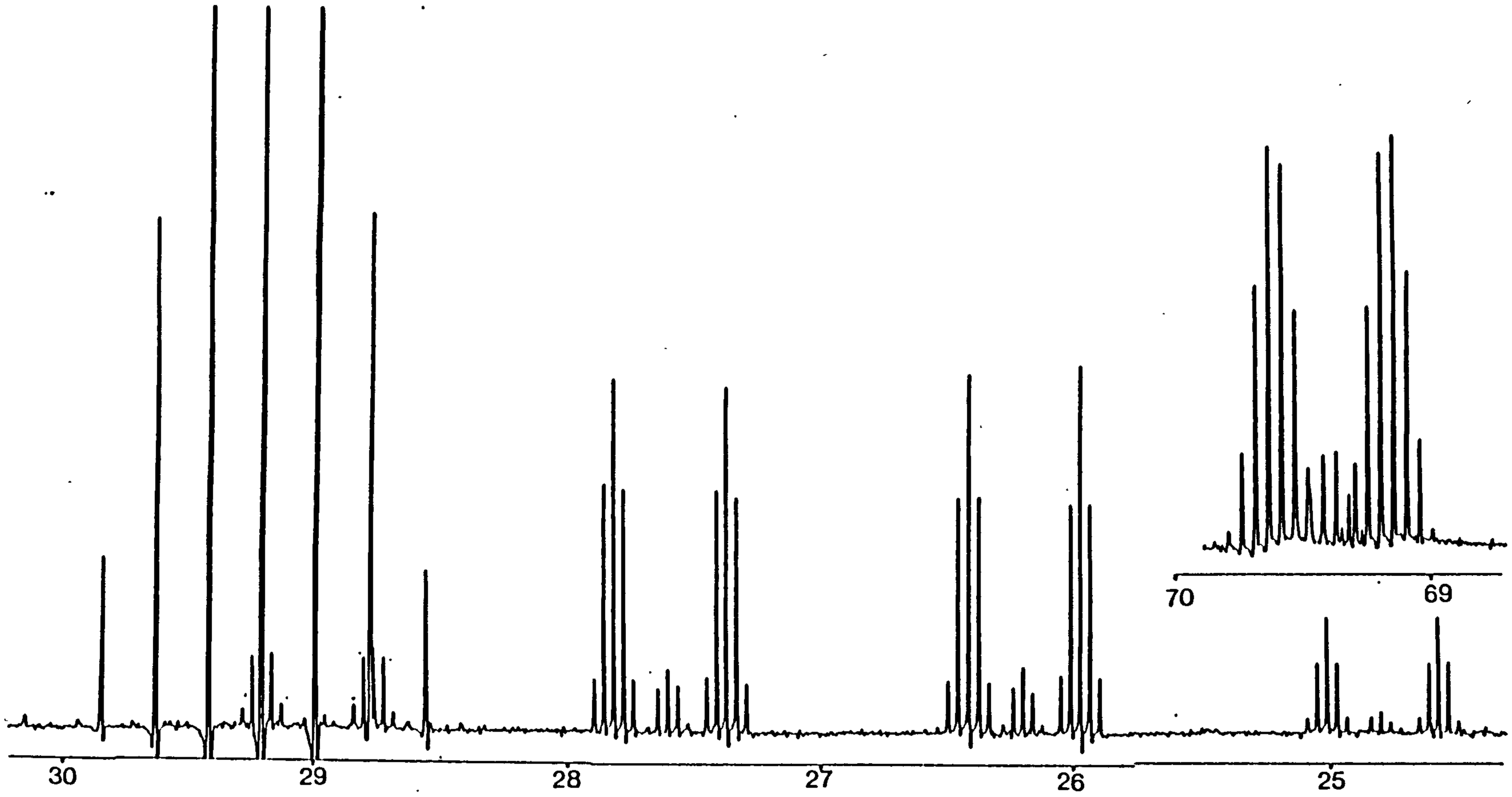


Figure 2.11 The fully proton coupled ^{13}C nmr spectrum of [3,3'-$^{13}C_2$]-β-hydroxy-β-methyl glutarate.

TABLE 2.7

Specific activity from incorporation studies using [^{14}C]β-hydroxy-β-methyl glutarate.

[3-^{14}C]PRECURSOR	YIELD (mg)	SPECIFIC ACTIVITY (dpm/mmol)	DILUTION
Di-sodium salt	24	9.32×10^6	4.2
Di-ethyl ester	20	2.74×10^6	14.4

with allyl bromide in a Grignard reaction to generate 4-hydroxy-4-methyl-hepta-1,6-diene (114) which in turn was converted to the diacid (115) by ozonolysis at -78°C, followed by oxidative workup. For incorporation studies, both these labelled forms, *ie.* ^{14}C and doubly labelled ^{13}C, of glutarate were converted to the di-sodium salt. The ^{14}C labelled glutarate was also converted to the corresponding diethyl ester. All the compounds were analysed by ^{1}H nmr spectroscopy and were found to be identical to authentic spectra. The fully proton coupled ^{13}C nmr spectrum of [2,3-^{13}C]-β-hydroxy-β-methyl glutarate was obtained and is shown in figure 2.11 and illustrates the couplings between the labelled carbons and directly attached and remote hydrogens.

In order to determine the optimum feeding conditions for the ^{13}C labelled glutarate, the ^{14}C labelled glutarate was fed to the growing second stage culture in two separate experiments, as its di-sodium salt (250mg, 1.2mmol) and as its diethyl ester (250mg, 1.15mmol). The isolated enriched metabolites was analysed by tlc against a standard sample of methyl pseudomonate and the specific activity of the isolated methyl pseudomonate was then determined by liquid scintillation counting. The results from these two incorporation experiments (table 2.7) show that this ^{14}C labelled HMG was incorporated with higher efficiency into pseudomonic acid as the di-sodium salt. To locate the actual sites of labelling the [2,3-$^{13}C_2$]HMG was fed as the di-sodium salt and the resultant enriched metabolite was analysed by ^{13}C nmr.

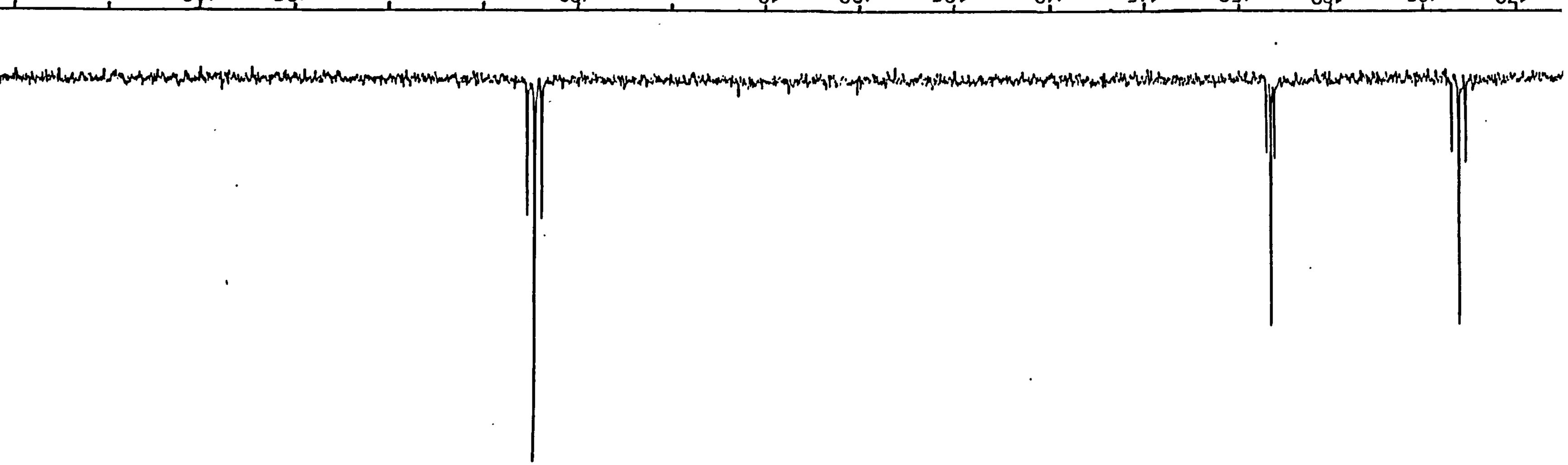


Figure 2.12 (cont)

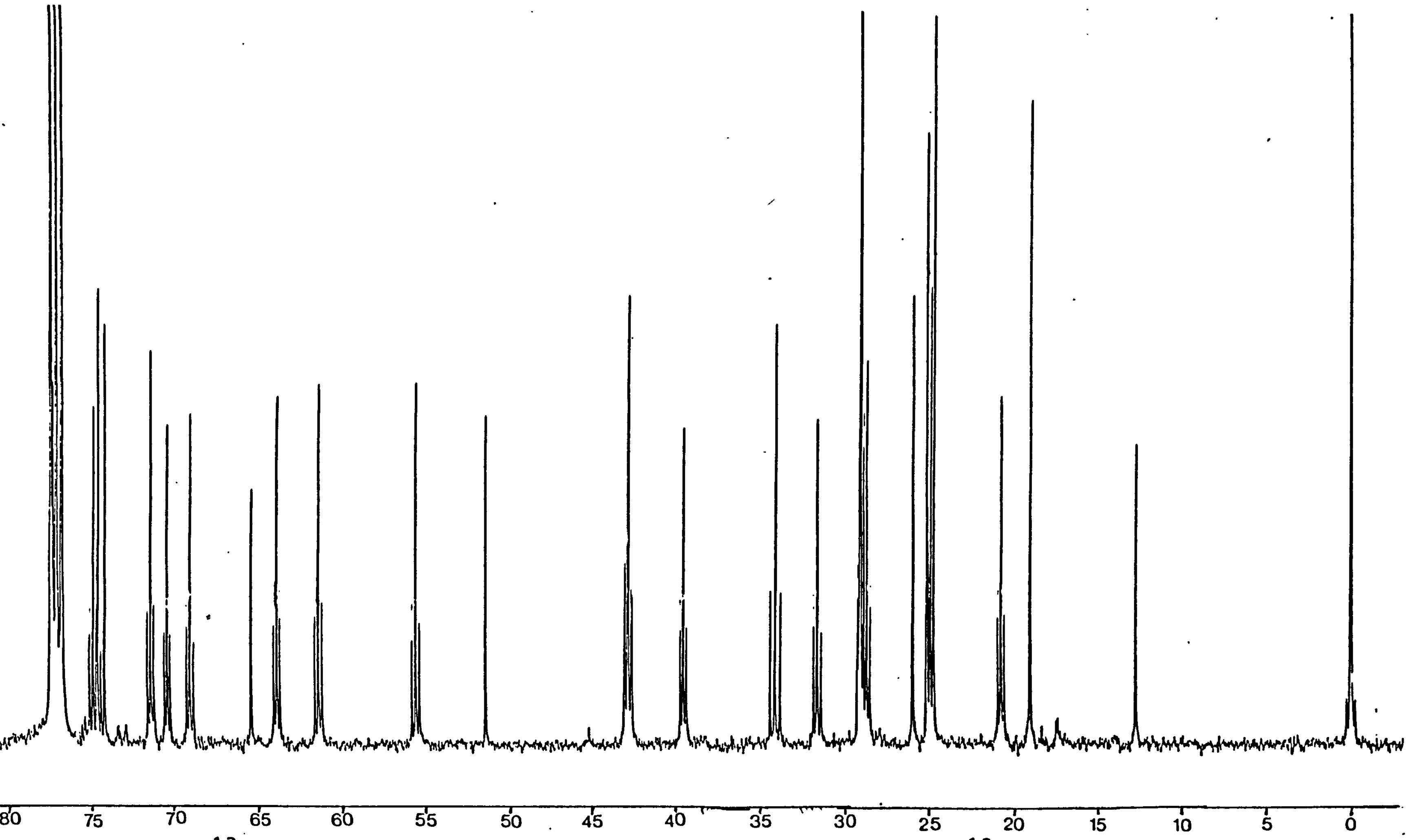
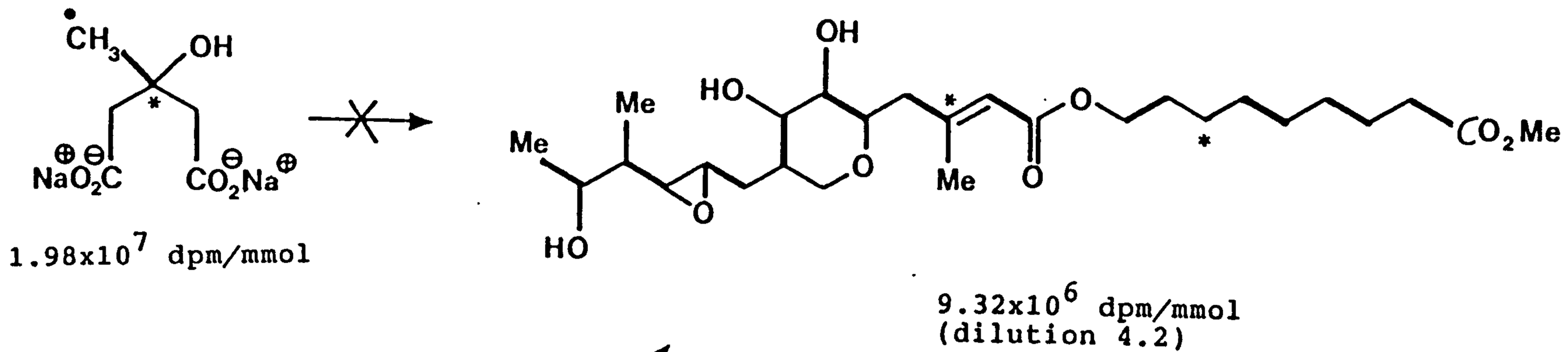


Figure 2.12 ^{13}C nmr spectrum of methyl pseudomonate labelled by [2,3-$^{13}C_2$]-β-hydroxy-β-methyl glutarate

TABLE 2.8:

^{13}C enrichment observed in the 100.61MHz ^{13}C nmr spectrum of methyl pseudomonate enriched from [3,6-$^{13}C_2$]β-hydroxy-β-methyl glutarate feed.

CARBON	δ_{CC} (ppm)	J_{CC} (Hz)	CARBON	δ_{CC} (ppm)	J_{CC} (Hz)
1	167.99	77	14	20.94	39.8
2	118.39	77	15	19.22	____
3	157.69	41.6	16	65.83	____
4	43.17	41.4	17	12.80	____
5	75.37	42.3	1′	175.79	58.7
6	69.44	42.4	2′	34.33	58.5
7	70.81	36.7	3′	25.08	35.8
8	39.79	36.6	4′		
9	31.84	45.4	5′	29.27	signals
10	55.95	45.3	6′		overlap
11	61.71	44.3	7′	26.13	____
12	43.10	44.2	8′	28.88	39.1
13	71.60	39.8	9′	64.25	39.1



●^{13}C
*^{13}C or ^{14}C

Scheme 2.6

An intial incorporation experiment with the doubly ^{13}C-labelled di-sodium HMG was preformed using the intial set of fermentation and subculturing conditions. The yield of the isolated, enriched metabolite was poor - 10mg as a colourless oil, and the ^{13}C nmr spectrum showed ^{13}C-^{13}C coupling satellites throughout. This indicated that the precursor had not been incorporated intact, but had been taken up by the cells and degraded to acetyl CoA and subsequently incorporated into the final metabolite. There was no evidence for enhanced enrichment at C-3, C-4 and C-7', the expected sites of labelling had the precursor been incorporated intact. This experiment was repeated after the problems with the fermentation and metabolite production had been resolved. Using the modified fermentation conditions, di-sodium [2,3-$^{13}C_2$]HMG was fed to a growing second stage culture. This time the production of the metabolite was good (43mg) and the ^{13}C nmr spectrum (figure 2.12) again showed ^{13}C-^{13}C coupling satelites throughout the molecule and these are summarised in table 2.8. The level of ^{13}C enrichment was much better than that achieved intially, but there was still no evidence for preferential enrichment of C-3, C-4 and C-7', required by the previously proposed pathway, (scheme 1.11). Hence, the observed incorporation of label from β-hydroxy-β-methyl glutarate into pseudomonic acid is entirely *via* prior breakdown to acetyl CoA and subsequent re-incorporation (scheme 2.6), and so HMG does not appear to be directly involved in pseudomonic acid biosynthesis.

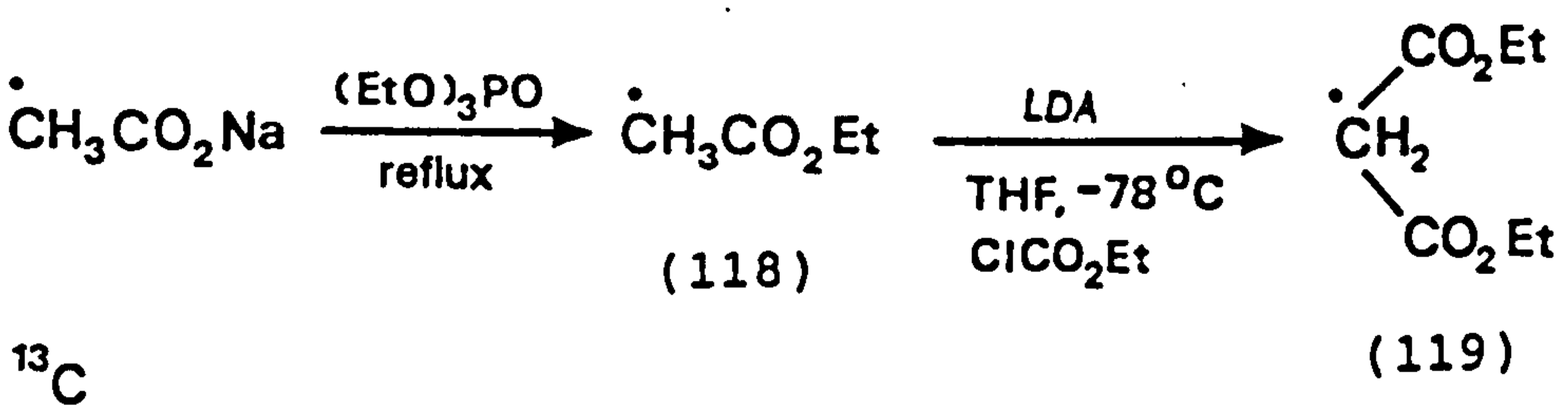
Recently, other workers[32a,b] have discussed results using

^{14}C labelled HMG which they interpret in favour of intact incorporation. However, they do not actually locate the actual labelled sites in the isolated enriched metabolite. These workers, Mantle *et al* [32a], in their incorporation studies, used the fermentation conditions detailed in the original paper[16] on pseudomonic acid biosynthesis. Their evidence for intact incorporation of glutarate was based on percentage incorporation of ^{14}C label into pseudomonic acid from [3-^{14}C]-β-hydroxy-β-methyl glutarate (HMG). They found a 0.9% incorporation of HMG and compared this value to that obtained from an incorporation study using [1-^{14}C]acetate. HMG incorporation was approximately twice that found for [1-^{14}C]acetate which suggests that HMG is a more specific precursor than acetate which provides most of the carbon atoms in pseudomonic acid. In further studies[32b], [3-^{14}C]HMG was fed to a *P. fluorescens* culture to which ethionine had been added. Ethionine serves to inhibit pseudomonic acid biosynthesis and causes monic acid A to be produced. Interestingly, this isolated monic acid A was not significantly labelled with ^{14}C. This finding was explained by suggesting that this monic acid represents late synthesised compound constructed from late synthesised precursors which are excess to biosynthetic demand as pseudomonic acid biosynthesis ceases at the end of fermentation. If this monic acid A had been a degradative product from pseudomonic acid, then it should have shown a similar specific radioactivity after adminstration of [^{14}C]HMG - as described previously. The ^{14}C label from [^{14}C]HMG fed in this experiment was accounted for in the folllowing manner; a small amount 7.6 - 7.7% was located in

the broth, and the remainder was assumed to have been largely cell associated or lost as $^{14}CO_2$ during the fermentation. This finding detracts rather than substantiates the earlier conclusion found by these workers.

2.7 SYNTHESIS AND INCORPORATION OF [2-^{13}C]MALONATE

In order to obtain more information on the biosynthetic origns of these carbon atoms which had been predicted to be derived from HMG, *ie.* C-3, C-4 and C-7′ in pseudomonic acid, the role of malonate in this biosynthetic pathway was investigated. Malonyl-CoA derived from carboxylation of acetyl-Co A is the basic chain extension unit in both fatty acid and polyketide biosynthesis. Incorporation of [2-^{13}C]malonate and observation of differential levels of labelling could provide information on "starter" groups and chain extension processes. Previous workers, Mellows *et al* [16], did not investigate the role of malonate in this pathway but found that the idea of propionyl-CoA as the 'primer' and chain extension by three malonate units did not fit the observed labelling from [1-^{13}C]-, [2-^{13}C]- and [1,2-^{13}C]acetates. By this theory, C-9′ would necessarily have to be derived from C-2 of acetate and not from C-1 as found, and the chain extension does not fit simple acetate plus malonate additions since there is a change in direction of addition of the acetate units between C-9′ and C-8′. Also, the level of enrichment at C-7′ is significantly less than that observed elsewhere in the enriched metabolite. The anomaly of label from [1-^{13}C]propionate going solely into C-7′ could not be explained either, by these workers.


•CH3CO2Na
(EtO)3PO
reflux
•CH3CO2Et
LDA
THF, -78°C
ClCO2Et
CO2Et
•CH2
CO2Et
•13C
(118)
(119)

Scheme 2.7

They proposed that the 'primer' for this C_9 moiety might be β-hydroxy-β-methylglutarate, a theory for which no supporting evidence has been found in the present work.

Diethyl [2-^{13}C]malonate was synthesised[33] as shown in scheme 2.7. As with the synthesis of the labelled HMG precursors, the synthetic route was first optimised using unlabelled materials. This synthesis again starts from [2-^{13}C]acetate which is converted to ethyl [2-^{13}C]acetate (118) by an ester exchange reaction with triethyl phosphate[31]. Lithium di-isopropylamide (LDA) was then used to generate the enolate of the labelled ethyl acetate, which was then condensed with ethyl chloroformate at -78°C, so generating the labelled diethyl malonate (119). Again, all intermediate compounds in this synthetic route were analysed by ^{1}H nmr spectroscopy. All were identical to spectra obtained from authentic material. The ^{1}H nmr spectrum of diethyl [2-^{13}C]malonate also indicates the degree of ^{13}C labelling. Since, 90 atom % sodium [1-^{13}C]acetate was used in this synthesis there was a small residual signal for $^{12}CH_2$ at δ2.0 and a much more intense doublet due to ^{13}C coupling to ^{1}H for $^{13}CH_2$. For incorporation studies di-sodium malonate was also synthesised, by base hydrolysis of diethyl malonate. The authenticity of this labelled malonate was checked by infra red spectroscopy. The infra red spectrum showed bands for carboxylate ions rather than ester carbonyl stretching bands, indicating that the ester groups had been hydrolysed. The high field ^{1}H nmr spectroscopy showed no ester signals and indicated that the degree of labelling was approximately

TABLE 2.9

^{13}C enrichment observed in the ^{13}C nmr spectrum of methyl pseudomonate

CARBON	δ_C (ppm)	^{13}C ENRICHMENT[a]	^{13}C ENRICHMENT[b]
2	117.8	2.0	3.0
4	44.0	1.9	2.1
6	74.2	1.9	2.6
8	35.1	1.9	2.8
10	55.5	2.0	3.1
12	42.8	1.9	2.1
14	20.6	1.8	2.4
15	18.9	2.0	2.9
2′	34.0	2.3	3.5
4′	29.0	2.0	3.8
6′	29.0	2.0	3.8
8′	28.7	2.1	3.9

a 250mg [2-^{13}C]malonate (100.61MHz ^{13}C nmr spectrum)
b 1.07g [2-^{13}C]malonate (75.47MHz ^{13}C nmr spectrum)

90%.

Using the modified fermentation conditions, as detailed previously, diethyl [2-^{13}C]malonate (250mg, 1.6mmol) was fed to a growing second stage culture after 20hrs fermentation. Production of the metabolite was good (50.5mg), and this was analysed by high field ^{13}C nmr for ^{13}C enrichment. However, when this ^{13}C nmr spectrum was compared with a natural abundance ^{13}C nmr spectrum determined under exactly the same experimental conditions as used for the enriched sample, there was no observed enrichment at all. One possible reason for this lack of observable incorporation could be a transport problem since *P. fluorescens* is a Gram negative bacterium, and consequently transport problems across the cell wall and membranes, to the site of synthesis inside the cells, must be considered. To try to solve this possible transport problem, the [2-^{13}C]malonate was re-fed as the di-sodium salt, allowing for the possibility of the malonate in this charged form, being able to enter the cells *via* the charged pores through which salts normally pass into the cells. The modified culture and fermentation conditions (as described previously) were used in this incorporation study. Again, production of the metabolite was reasonable, and the high field ^{13}C nmr spectrum of this isolated metabolite was determined and this time it did show observable incorporation of ^{13}C label into the metabolite. The levels of enrichment at the various sites in the isolated methyl pseudomonate are detailed in table 2.9. These were calculated by comparing the intensities of the lines in the natural abundance ^{13}C nmr spectrum with the intensities

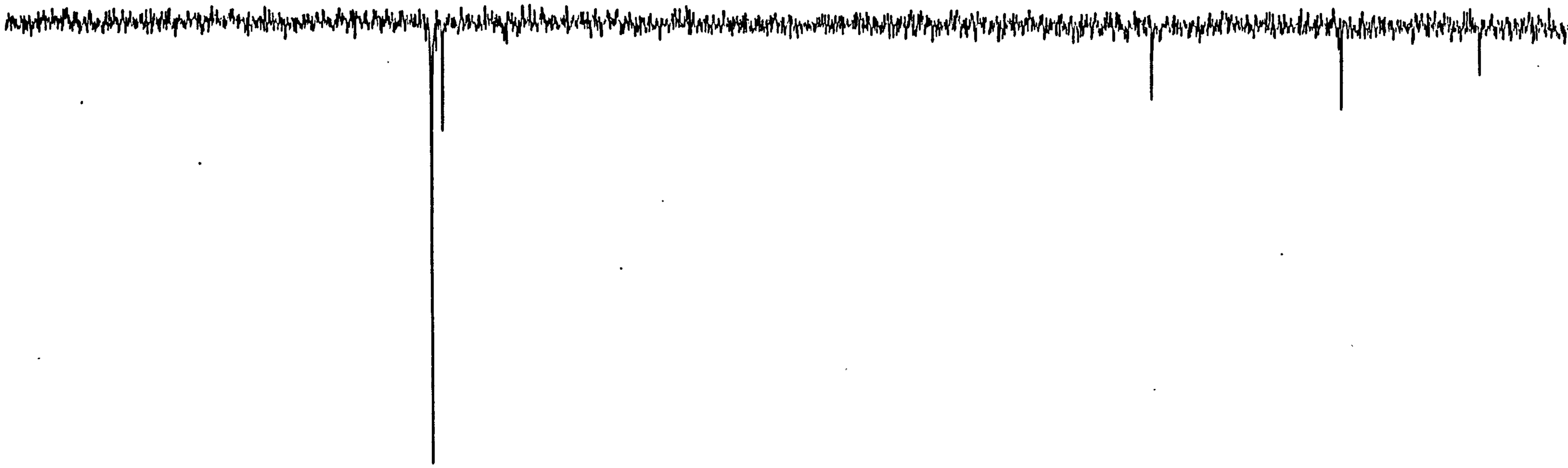


Figure 2.13 (cont) δ_C (PPM)

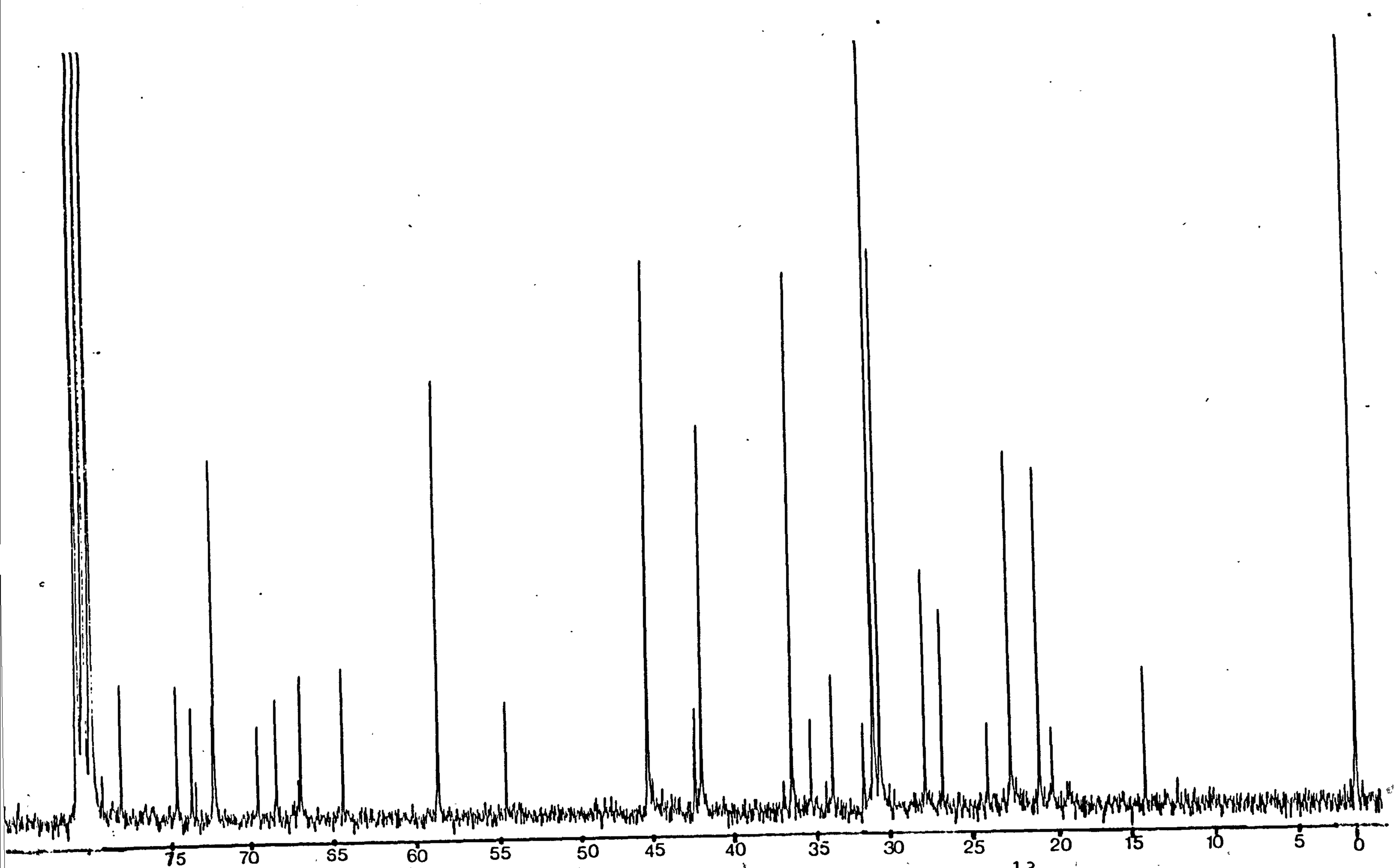
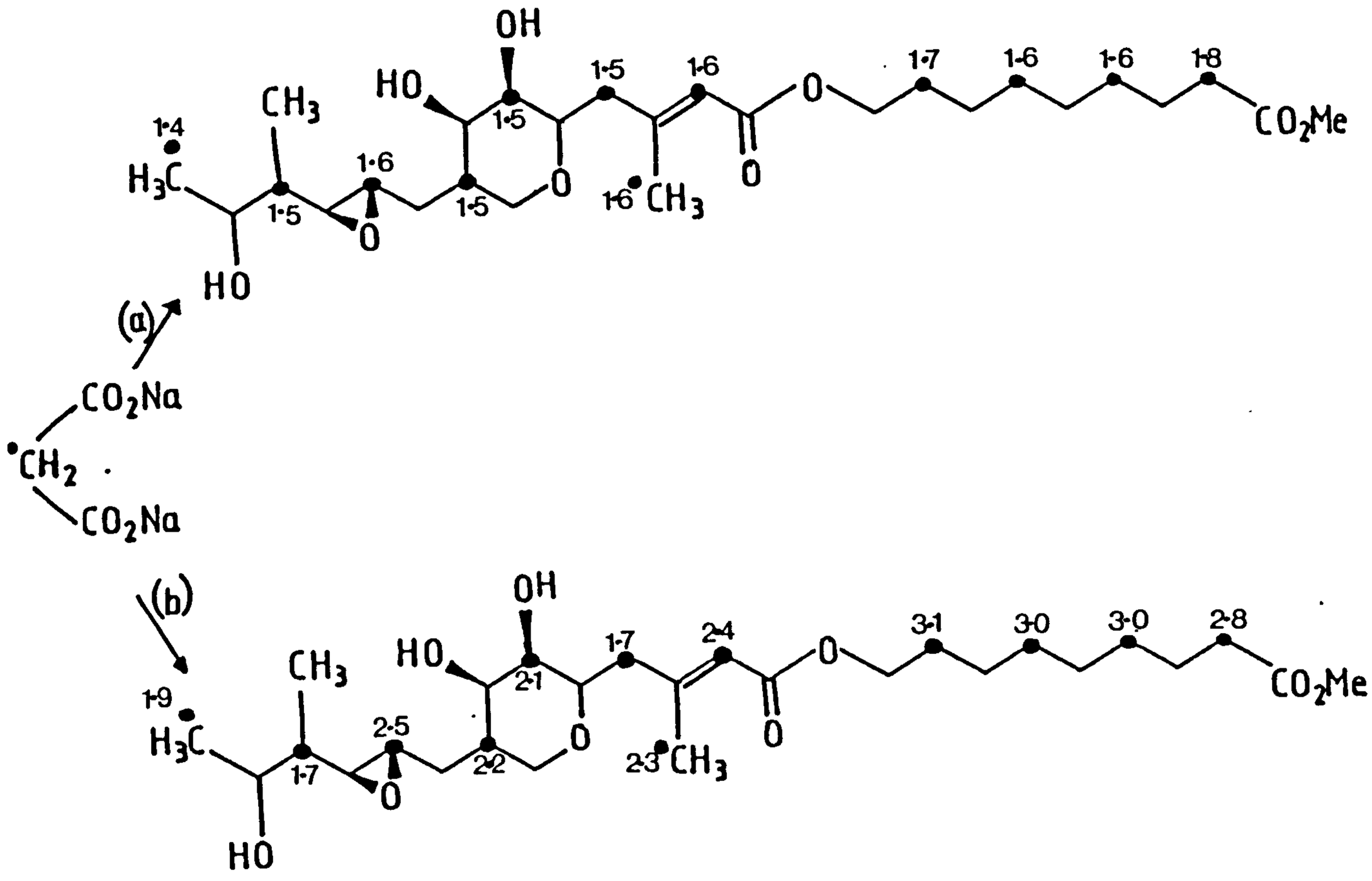


Figure 2.13 ^{13}C nmr spectrum of methyl pseudomonate labelled by sodium [2-^{13}C]malonate.

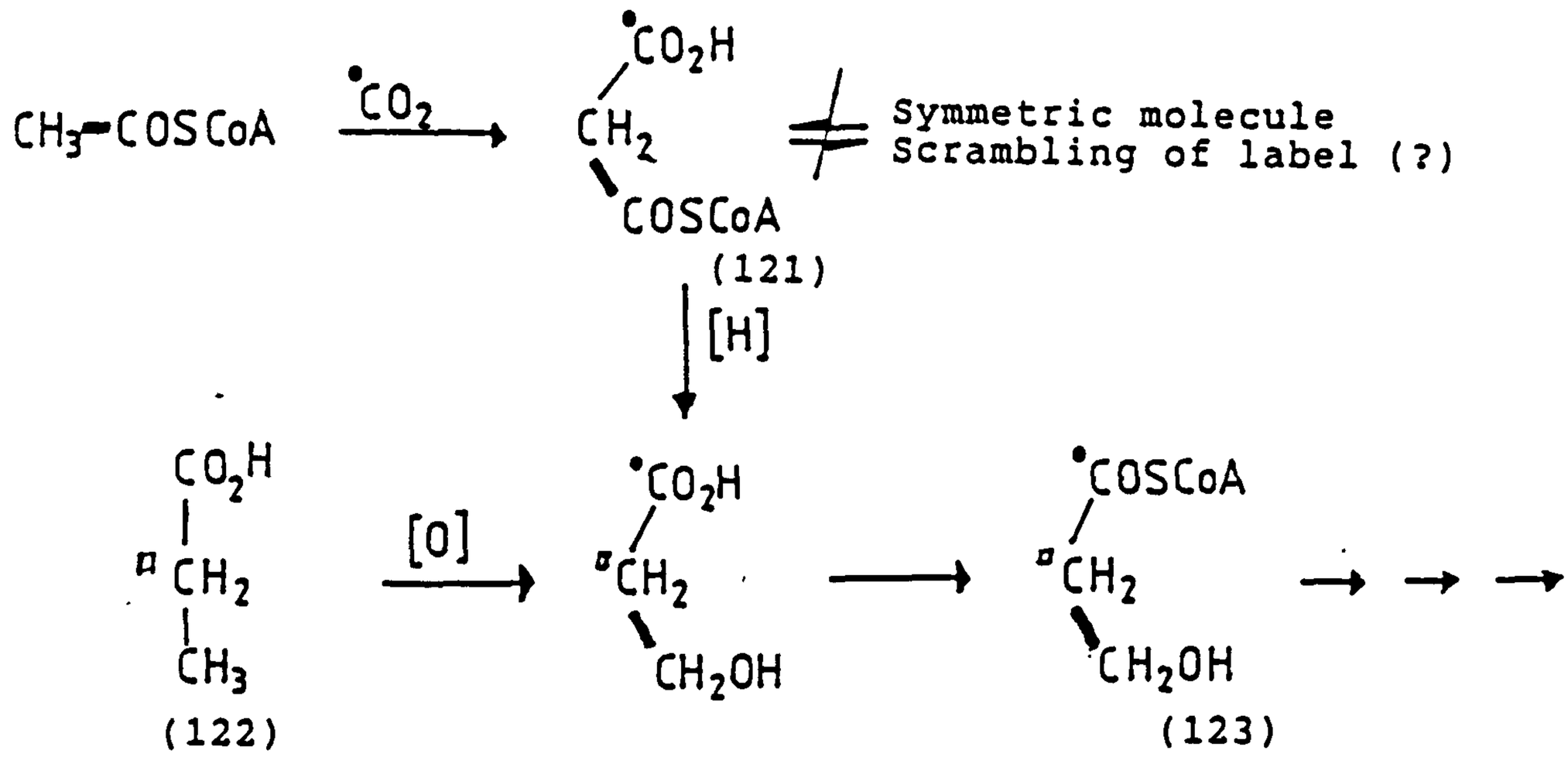


Scheme 2.8

(a) 250mg [2-^{13}C]malonate
(b) 1.07g [2-^{13}C]malonate

observed in the ^{13}C nmr spectrum of the enriched sample, all these spectra were obtained under the same experimental conditions, and these enrichment levels were then normalised with the average intensity of all the known un-enriched signals. This method for quantifying ^{13}C enrichment is not necessarily the best and the problems associated with trying to quantify ^{13}C enrichment are well reviewed by Simpson[26].

Scheme 2.8 illustrates the observed enrichment levels, and from these, all sites labelled from the methyl carbon from acetate were also labelled equally from malonate. There was no significant enrichment at any site indicating "starter" unit effects. This experiment was repeated, but this time the precursor was fed at a much higher level, to see if differential incorporation could be observed; 1.07g (7.2mmol) of malonate was fed as a solution in sterile water to 125ml of a growing second stage culture, compared to 1.7mmol of malonate to 250ml of second stage culture, used previously. Methyl pseudomonate (30mg) was isolated from this experiment and was analysed by ^{13}C nmr. The ^{13}C nmr spectrum obtained is shown in figure 2.13 and evidence for differential incorporation was obtained. The ^{13}C enrichment levels were calculated from line averaged intenties from two sets of acquired data, ie. the ^{13}C nmr spectra of the natural abundance and enriched sample were obtained twice. This was done to compensate for the random variation in intensities in the spectral lines, even although the spectra were obtained from the same sample, under the same experimental parameters. The ^{13}C enrichment was then calculated by dividing the average line intensity of the enriched sample by that of the natural abundance and then



(122) (123)

Scheme 2.9

normalising it with the average spectral line intensity from all non enriched signals. This ensures more reliable ^{13}C enrichment values, which are detailed in table 2.9. The ^{13}C enrichments levels are much higher in the 9-hydroxynonanoic acid side chain compared to the levels observed in the monic acid portion of pseudomonic acid, and these results are summarised in scheme 2.8.

From these observed enrichment levels, malonate does appear to play an important role in the biosynthesis of the 9-hydroxynonanoic acid moiety. Four intact acetate units appear to form this moiety, and one way of explaining this observation biosynthetically is illustrated in scheme 2.9. However, this theory does not account for the reduced enrichment of C-7′ and the incorporation of label from [1-^{13}C]propionate (122) solely into this position, C-7′. A possible further test would be to try incorporating 3-hydroxypropionate (123) into pseudomonic acid as this would be a precursor by this proposed route.

2.8 INCORPORATION OF Sodium [1-^{13}C]BICARBONATE

To try to account for the reduced enrichment of C-7′ from acetate, it was decided to see if this carbon atom could be derived from another source. Incorporation of ^{13}C-bicarbonate was tried to see if C-7′ was derived *via* this 'C-1 pool' as well as from acetate, and so could account for the observed reduced enrichment level. The chalk used to buffer the second stage medium (required to limit glucose uptake by the bacterium because if glucose utilization is

too rapid the bacteria quickly die) was replaced by ^{13}C labelled bicarbonate. Unfortunately, the pH of the culture supernatant had fallen to 4 during the course of the fermentation, and this is unusually low. Isolation of the metabolite was carried out in the normal way and 20mg of a colourless oil was obtained. The ^{13}C nmr of this isolate showed it to be methyl pseudomonoate but when compared with a natural abundance ^{13}C nmr spectrum, determined under identical conditions, no enrichment was observed. Consequently, nothing more can be said from this negative result as to the origin of C-7′ and to the role of malonate in pseudomonic acid biosynthesis.

2.9 INCORPORATION OF SODIUM [1-^{13}C]ACETATE

It was decided to check the previous workers[16] findings, that feeding [1-^{13}C]acetate resulted in reduced enrichment at C-7′, since this has important implications for the role of malonate in this biosynthetic pathway. Sodium [1-^{13}C]acetate (200mg, 2.4mmol) was fed to a growing second stage culture, and production of the methyl pseudomonate was good (40mg). Analysis of the ^{13}C nmr spectrum of this enriched metabolite did reveal ^{13}C enrichment in all the expected sites (table 2.10). These were calculated as described previously for the di-sodium [2-^{13}C]malonate incorporation experiment. However, incorporation was at a much lower level (1.5x natural abundance) than that found by the previous workers[16] (7x natural abundance). In a repeat experiment to try to increase enrichment levels, the [1-^{13}C]acetate was pulse fed over a period of time, ie.

TABLE 2.10

^{13}C nmr enrichments observed in the 75.47MHz ^{13}C nmr from methyl pseudomonate enriched from [1-^{13}C]acetate.

CARBON	δ_C (ppm)	^{13}C ENRICHMENT[a]	^{13}C ENRICHMENT[b]
1	166.76	1.5	2.2
3	156.62	1.4	2.2
5	74.85	1.4	2.1
7	70.34	1.5	2.2
9	31.60	1.0	2.0
11	61.30	1.5	2.4
13	71.32	1.7	2.6
1'	174.34	1.7	2.8
3'	24.90	1.5	2.3
5'	29.00	1.3	1.6
7'	25.94	1.5	1.3
9'	63.82	1.4	2.1

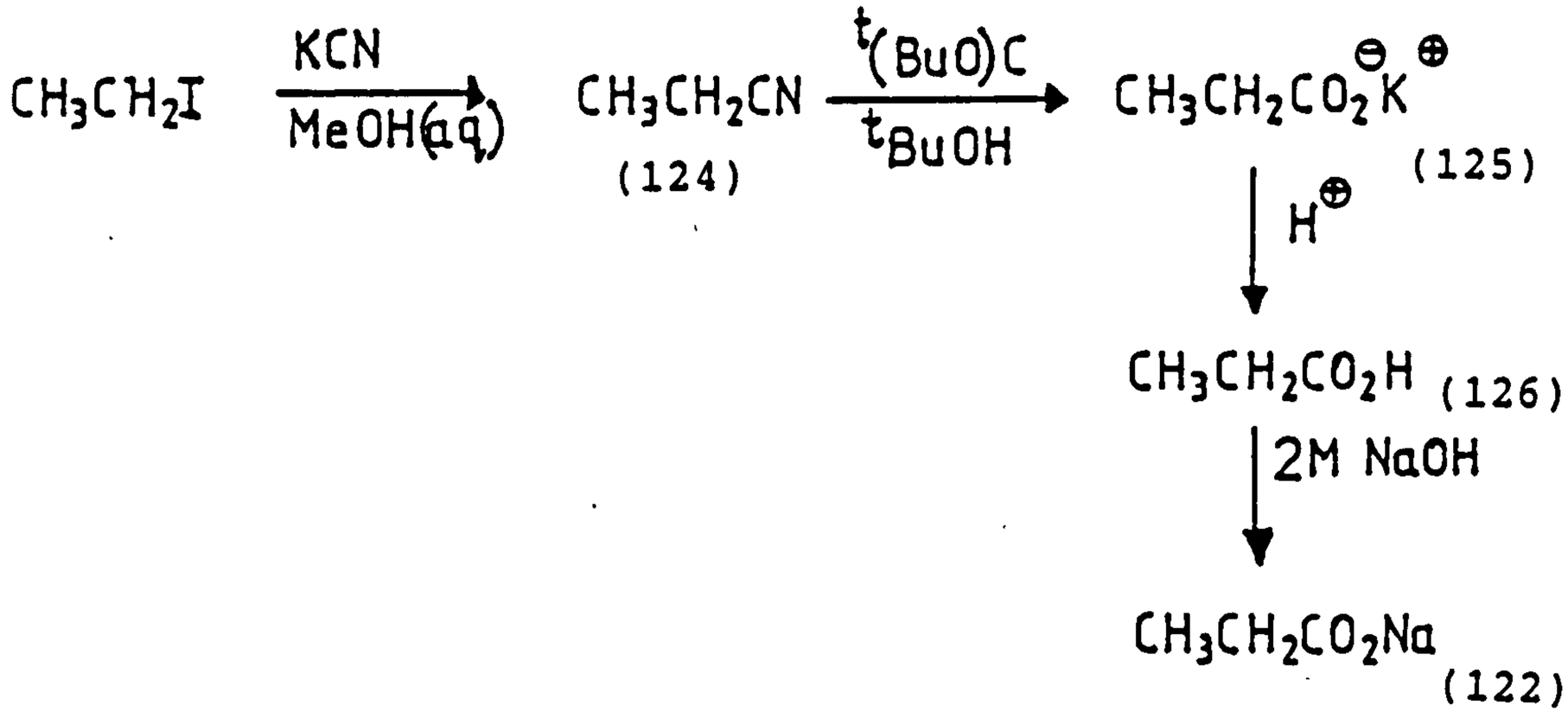
a 200mg [1-^{13}C]acetate
b 500mg [1-^{13}C]acetate pulse fed

100mg (1.2mmol) was added to 250mg of growing second stage culture after 10h, 14h, 22h, 26h and 30h. To prevent abnormal changes in the pH during the fermentation, resulting from these acetate feeds, the second stage medium was buffered on the alkaline side by increasing the chalk concentration and the amount of Na_2HPO_4 and reducing the amount of KH_2PO_4 present.

Analysis of the ^{13}C nmr spectrum of the isolated enriched sample, from this incorporation experiment, again showed all the expected sites to be labelled. Enrichment levels, which were calculated by the method as outlined for the previous experiment, were better (twice natural abundance) but still less than the observed enrichment levels found by previous workers[16]. However, C-7' was shown to be less enriched than the other sites (table 2.10). This confirms the earlier findings but does not give any more information which can be used to explain the observed enrichment pattern from [2-^{13}C]malonate.

2.10 SYNTHESIS OF Sodium [1-^{13}C]PROPIONATE AND DOUBLY LABELLED 9-HYDROXYNONANOIC ACID

For future studies, [1-^{13}C]propionate (122) and doubly labelled 9-hydroxynonanoic acid (3) will be required. The synthetic route to [1-^{13}C]propionate is based on Cane's[39] synthesis of [1-^{13}C,$^{18}O_2$]propionate. The synthetic route to 9-hydroxynonanoic acid was developed from literature preparations of the intermediate compounds, with the req-uirement to have a step for introduction of radioactive (^{14}C


CH3CH2I
KCN
MeOH(aq)
CH3CH2CN
(124)
t(BuO)C
tBuOH
CH3CH2CO2K
(125)
H⊕
CH3CH2CO2H (126)
2M NaOH
CH3CH2CO2Na
(122)

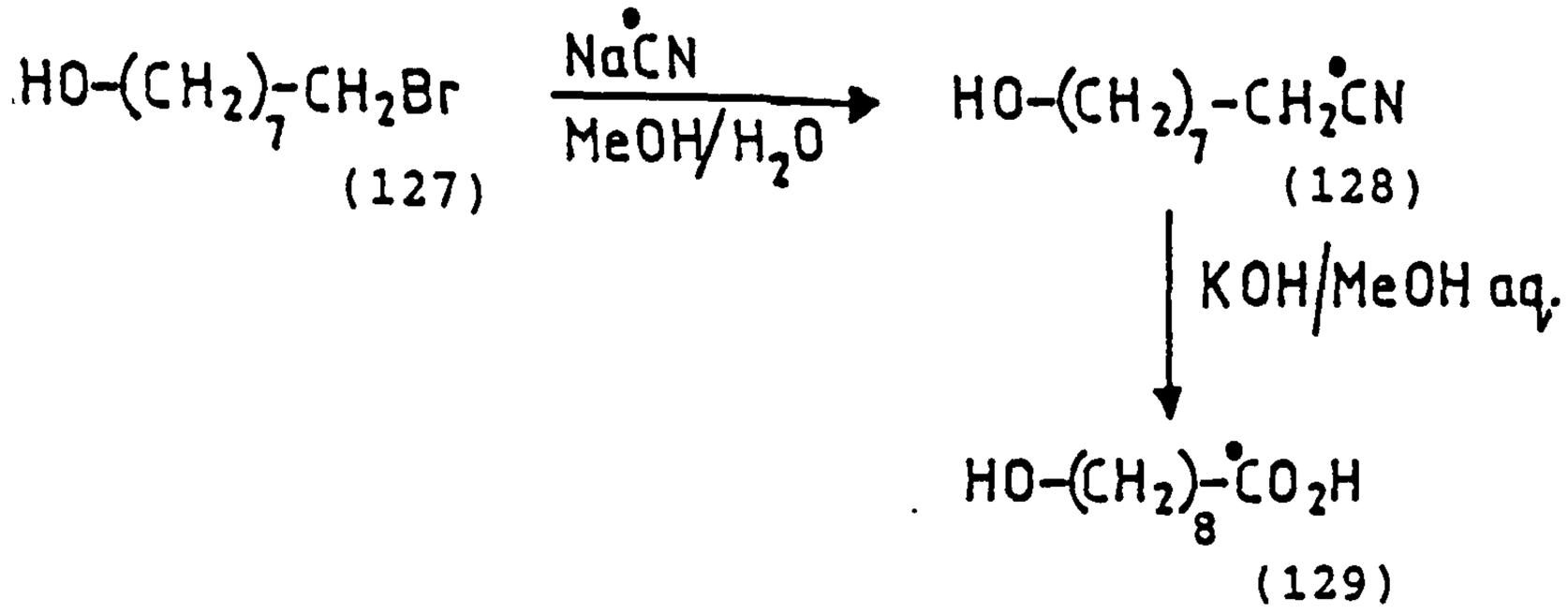
Scheme 2.10

and ^{3}H) label.

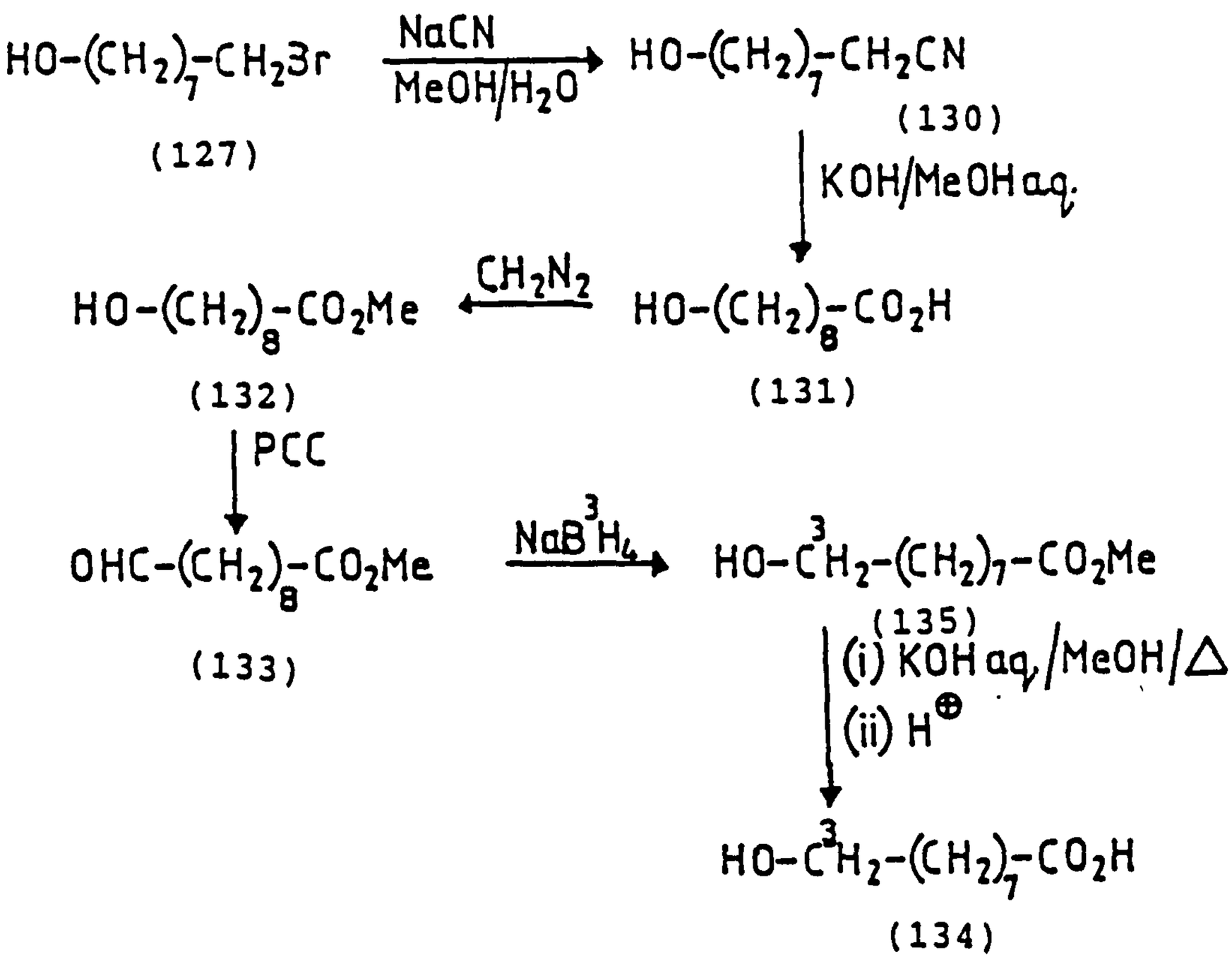
The incorporation of [1-^{13}C]propionate will be used to test the previous findings[16] that it is solely incorporated into C-7'. This is important since, the unusually low incorporation of [1-^{13}C]acetate into C-7', compared to the enrichment levels at all other labelled sites requires further investigation, especially in the light of the [2-^{13}C]malonate observed ^{13}C enrichments in the 9-hydroxynonanoic acid side chain.

To further test the theory that pseudomonic acid is synthesised from preformed C_{17} and C_9 subunits, the incorporation of 9-hydroxynonanoic acid into pseudomonic acid should be studied. This could be achieved by feeding 9-hydroxynonanoic acid radio-labelled with ^{14}C in the acid group and ^{3}H in the CH_2 group adjacent to the hydroxyl group. If this precursor is incorporated intact into pseudomonic acid then the ratio of $^{14}C/^{3}H$ of the precursor to the ratio observed in the isolated metabolite should be the same.

The synthetic route to [1-^{13}C]propionate (122) is shown in scheme 2.10. Step one of this synthesis involves the preparation of [1-^{13}C]propionitrile (124) from potassium cyanide and ethyl iodide in refluxing absolute methanol. This nitrile (124) was then hydrolysed by refluxing with ^{t}butoxide in ^{t}butyl alcohol. The potassium propionate (125) generated was then converted back to the free acid before being converted by titration with sodium hydroxide to the



Scheme 2.11



Scheme 2.12

required sodium [1-^{13}C]propionate (122). It should be noted that the original prepartion stated that this should be lyophilized, but if this is done then the free propionic acid generated is lost! The ^{1}H nmr spectrum of the [1-^{13}C]propionate again showed the coupling effects of directly bonded and remote hydrogens, the resonance for the methyl signal at δ1.0 is now a multiplet due to three bond ^{1}H-^{13}C coupling, and the methylene signal is also a multiplet due to two bond ^{1}H-^{13}C coupling.

The synthetic route to the doubly labelled 9-hydroxynonanoic acid is detailed in schemes 2.11 and 2.12. Scheme 2.11 indicates how the ^{14C} label is introduced into the acid group of 9-hydroxynonanoic acid and scheme 2.12 indicates how the ^{3}H label is put in place. This will be done to avoid carrying labelled material through such a long reaction sequence. The ^{14}C is first introduced as indicated in scheme 2.11 and then the synthesis is repeated with unlabelled potassium cyanide through to point where the ^{3}H label is introduced from sodium borotritiide (scheme 2.12), and the doubly labelled compound required for feeding would come from an equal mixture of ^{14}C labelled material (129) and the ^{3}H labelled material (135) - the ratio of ^{14}C/^{3}H being determined from this mixture.

This synthesis starts from 8-bromo-1-octanol (127) which is converted to the corresponding nitrile[35] (128) by reaction with potassium cyanide in aqueous methanol. The nitrile (128) was then hydrolysed[36] to 9-hydroxynonanoic acid (129) by reaction with potassium hydroxide in methanol. The ^{14}C

label will come from potassium [^{14}C]cyanide. The 9-hydroxynonanoic acid (131) was then treated with an excess of ethereal diazomethane (generated as detailed previously) to form 9-hydroxynonanoate (132), and this was then oxidised[37] to the corresponding aldehyde (133) by reaction with pyridinium chlorochromate (PCC). This was then re-reduced to the alcohol using sodium borohydride[38], and for the introduction of ^{3}H, sodium borotritiide would be used, and finally, hydrolysis of the resulting hydroxy ester compound (134) results in 9-hydroxynonanoic acid (135). ^{1}H nmr spectroscopy was used to determine the structures of the generated intermediate compounds. Before this route can be used to synthesise radio-labelled 9-hydroxynonanoic acid, the step which introduces the ^{3}H label will have to be optimised. The conversion yield of 29% is unacceptably low for a labelled synthesis.

2.11 FURTHER WORK

After optimising the re-reduction step, for the introduction of ^{3}H into the doubly labelled 9-hydroxynonanoic acid, incorporation studies could then be carried out and should provide more information on the biosynthesis of the 9-hydroxynonanoic acid side chain and its coupling to monic acid. Intact incorporation of this precursor would support the previous theory[16] of separate subunits that are synthesised shortly before being used to generate the final metabolite, whilst non-intact incorporation, might suggest that this side chain is synthesised by stepwise assembly intermediates onto monic acid. More information on the role

of malonate in this biosynthetic pathway might come from studying the incorporation of 3-hydroxypropionate, which would be a precursor if the proposed route outlined, (scheme 2.9), is correct. The 3-hydroxypropionate could be synthesised by a low temperature condensation reaction between ethyl formate and the enolate of ethyl acetate. A ^{13}C label could thus be conveniently introduced from sodium acetate by its conversion to ethyl acetate[31] which is subsequently used to synthesise the required 3-hydroxypropionate. The origin of C-7' could be further investigated by re-feeding [1-^{13}C]propionate to check the initial published result[16] and also determining the labelling pattern from [2-^{13}C]propionate, which could be synthesised by the method outlined by Nefkens and Zwanemburg[40]. This might also lend further information on the role of malonate in this pathway. It might also be worthwhile, testing to see if leucine plays any part in the biosynthetic pathway to pseudomonic acid. The role of leucine in terpenoid metabolism has been investigated by Overton *et al*[34]. These workers found that (3S)-3-hydroxy-methyl-glutaryl-COA (HMG CoA) from leucine breakdown was not incorporated directly into mevalonic acid (MVA) but acetyl CoA and acetoacetate produced by leucine breakdown, *via* HMG-CoA are subsequently incorporated into HMG-CoA and MVA. MVA is not a known precursor to pseudomonic acid but HMG-CoA was suggested as being one, even although no evidence for intact incorporation was found in this work. This result. is similar to these workers findings for terpene biosynthesis, but pseudomonic acid is basically polyketide in origin, so it would not be correct to draw too many similarities

between these two results. However, a trial incorporation of ^{14}C labelled leucine might produce some interesting findings.

GENERAL EXPERIMENTAL

General Experimental

Melting points were determined on a Kofler hot stage apparatus and were uncorrected. ^{13}C and ^{1}H nmr spectra were determined on various instruments, namely, Bruker WH400, Bruker WH360, Bruker WH300, WP200SY and WP80SY Fourier-transform Spectrometers, and were referenced on deuterated solvent, or on TMS. In the case of ^{2}H nmr spectra these were referenced on chloroform. Routine ^{1}H nmr spectra were obtained on a Varian EM390 or on a Joel JNM-PMX 60 continuous wave spectrometer. Mass spectrometry was carried out on a Kratos MS 50, or AEI MS 902 spectrometer, with ionisation being achieved by electron impact. Optical rotations were recorded on a Perkin Elmer 141 polarimeter. Infra red spectra were recorded on a Perkin Elmer 781 spectrophotometer and were referenced against the polystyrene absorption at $1603cm^{-1}$. Gas chromatography was carried out on a Pye 204 chromatograph.

Preparative thin layer chromatography was carried out on glass plates (20 x 20 cm), coated with a 0.5mm layer of silica gel (Fluka 60765 Kieselgel Gf_{254}). Bands were visualised using ultra violet light (254nm). Flash chromatrography was carried out on silica gel 400 - 200 mesh (Kieselgel 60 Merck).

Scintillation counting was carried out on a Beckmann LS7000 liquid scintillation counter. Programme four was used without automatic quench correction, butyl PBD (10g/l) in

methanol-toluene (50:50) was used as the scintillant. The counting efficiency was determined by using both standard channels ratio and H-number quench curves.

All solvents were of commercial grade and used without purification unless stated otherwise. Purification and drying of solvents was carried out by the procedures outlined by Perrin and Armarego, in " Purification of Laboratory Chemicals[62]". A dry N_2 atmosphere was obtained by passing compressed N_2 gas through a series of traps containing;

(i) concentrated sulphuric acid

(ii) glass wool

(iii) self-indicating silica gel.

EXPERIMENTAL

Chapter two

Experimental

FERMENTATION OF *P. FLUORESCENS* (NCIB 10586) AND ISOLATION OF PSEUDOMONIC ACID

FERMENTATION MEDIUM 1

The bacterium was maintained on nutrient agar slopes, stored at $4^{o}C$ and subcultured every 2 months. For inoculation, a slope was flooded with nutrient broth, 1ml of which was added to a seed stage flask. This seed stage medium (used to obtain good bacterial growth) consisted of; Oxoid nutrient broth (13g), peptone (5g), Na_2HPO_4 (2.6g), KH_2PO_4 (2.4g), and glucose monohydrate (1.1g), made up to 1l with distilled water. The pH of the medium was adjusted to 7.0 with 2M NaOH, dispensed into 500ml flasks (100ml/flask) and sterilised at $121^{o}C$ and 15lb in^{-2} pressure for 15 min). The culture was incubated at $26^{o}C$ for 24h. A sample (10ml) of this seed stage served as inoculum for the second (production) stage medium (100ml/500ml flask) which consisted of; KH_2PO_4 (0.4g), Na_2HPO_4 (0.65g), KCl (0.5g), $MnCl.2H_2O$ (0.003g), $MgSO_4.7H_2O$ (0.375g) and ground nut meal (21g) made up to 1l with distilled water. Again, the pH was adjusted to 7.0 prior to sterilisation at $121^{o}C$ and 15lb in^{-2} for 15min). Immediately after inoculation a sample (10ml) of 5% glucose in water (separately sterilised at 15lb in^{-2} for 15min) was added. Incubation was carried out at $26^{o}C$ for 24h on a rotary shaker.

FERMENTATION MEDIUM 2

For inoculation of the seed stage flasks using this set of

fermentation conditions, one culture slope of *P. fluorescens* was flooded with nutrient broth. A sample (1ml) of this, was added to a seed stage flask (50ml/250ml flask) containing yeatex (28.5g), glucose (1.1g), Na_2HPO_4 (2.6g), KH_2PO_4 (2.4g), and $(NH_4)_2SO_4$ (5.0g), made up to 1l with distilled water (the pH adjusted to 7.0 with 2M NaOH before sterilisation at 121°C and 15lb in^{-2} for 15 min). This culture was incubated at 25°C for 24h on a rotary shaker. 1ml of this seed stage was then used to inoculate each second (production) stage flask (25ml/250ml flask) containing; Arkasoy 50 (20g), corn steep liquor (5.0g), $(NH_4)_2SO_4$ (5.0g), $MgSO_4.7H_2O$ (0.5g), Na_2HPO_4 (1.0g), KH_2PO_4 (1.5g), antifoam (L-81) (a few drops), $CaCO_3$ (6.25g/1l) and glucose (60g) made up to 1l with distilled water and the pH again adjusted to 7.0 before sterilisation at 117°C, 15lb in^{-2} for 15min. This medium was stirred constantly when it was dispensed into the flasks to ensure even distribution of the chalk. The culture was incubated at 22°C for 48h on a rotary shaker after which time production was essentially complete.

MODIFIED CULTURE CONDITIONS

Due to the decline in production of the metabolites, the storage and fermentation conditions had to be modified. The following changes were made:

(1) Instead of maintaining the culture on nutrient agar slopes a nutrient deficient agar was used which slowed down any active growth and ageing of the

bacterium on the slopes. These slopes are viable for 3 months.

(2) The culture was not subcultured. After 3 months, a fresh set of working slopes were made up and inoculated from a freeze-dried isolate of the bacterium.

(3) In the second (production) stage of the fermentation, 'baffle' or 'spiked' 250ml flasks were used in place of Erlenmeyer flasks. These flasks increase the degree of aeration afforded to the growing culture by disrupting the circular motion of the liquid as it is shaken on the rotary incubator. This results in increased titres of the metabolite.

HPLC ANALYSIS OF CULTURE FILTRATES

A Waters μ-bondapak C-18 column was used for this analysis. The eluting solvent was methanol/0.05M ammonium acetate (3:2, v/v), pH 4.5. The flow rate was 1.5ml/min and uv detection was at 230nm. Pseudomonic acid A has a retention time of 6min and pseudomonic acid B has a retention time of 5.5min. under these conditions.

ISOLATION AND PURIFICATION OF METHYL PSEUDOMONATE FROM FERMENTATION MEDIUM 2

The second stage flasks were harvested as follows:

The cells were removed by centrifugation (10 000g) at 2°C. The pH of the combined supernatants (ca 250ml) was then adjusted to 4.5 with 2M HCl, saturated with NaCl and

extracted with 100ml of ethyl acetate. The layers were separated and the aqueous layer extracted with ethyl acetate (4x 100ml). The organic extracts were combined, dried over anhydrous $MgSO_4$ and the solvent evaporated *in vacuo* to yield a yellow/brown oil. This was dissolved in 5ml methanol and treated with an excess of ethereal diazomethane. This was stirred at room temperature for approximately 2h and after this time, any remaining diazomethane was removed by bubbling N_2 through the solution. The solvent was evaporated *in vacuo* to yield an oil which was purified by preparative tlc (solvent system; ethylacetate/ethanol, 40:1). The band at *rf* 0.3 afforded methyl pseudomonate as a colourless oil which crystallised on standing in the fridge. This showed signals at, δ_H ($CDCl_3$, 360.13MHz) 0.95 (3H, d, J=5Hz, 17-C$\underline{H}_3$), 1.20 (3H, d, J=5Hz, 14-C$\underline{H}_3$), 2.2 (3H, s, 15-C$\underline{H}_3$), 2.3 (2H, t, J=5Hz, 2'-C$\underline{H}_2$), 3.65 (3H, s, ester C$\underline{H}_3$), 4.1 (2H, t, J=5Hz, 9'-C$\underline{H}_2$), 5.75 (1H, s, 2-C$\underline{H}$). The remainder of this spectrum was complex but identical to a spectrum of authentic methyl pseudomonate.

SYNTHESIS OF DIAZOMETHANE

This general preparation gives a yield of 60%, ie. 0.03M solution in diethyl ether. This preparation was carried out using glassware which had no ground glass joints.

To a solution of ethanol (18ml) and diethyl ether (15ml) was added KOH (3g) in water (5ml), and this was warmed on a water bath to 60-70^{o}C until the ether starts to distil. At

this point, a solution of Diazald (10.75g) in diethyl ether (70ml) was added in portions over a period of 15 to 20 min. The diazomethane distilled over and was collected in a flask cooled in an ice-bath. Distillation was continued until the distillate was colourless. More diethyl ether was added to the reaction flask as required. The quantities of Diazald used in the preparation of the excess ethereal solution of diazomethane were reduced in portion to the yield of the isolated pseudomonic acid being methylated.

GROWTH PRODUCTION STUDIES

In all these studies, fermentation medium 2 was used, and inoculation procedures from culture slope to seed stage flask and from seed stage flask to second stage flasks were as described previously. In each study, one first stage flask was grown up, and used to inoculate ten second stage flasks. At timed intervals, 0.1ml of culture fluid was removed from all ten flasks, combined and after micro-filtration was analysed by hplc against a standard sample of pseudomonic acid of known concentration. Hplc assay conditions were as previously described. Initial production of the metabolites occurs after 20h fermentation and reaches a maximum after 60h fermentation. Repetition of this experiment confirmed this result.

After modifying the culture maintenance and fermentation conditions, the production of the pseudomonic acids with time was checked again. The following changes were made in this growth production study:

(1) The second stage medium was contained in 'baffled' 250ml flasks.

(2) The pH of the culture fluid removed at the set time intervals (0.1ml/flask) was adjusted to 8 before the cells were removed by centrifugation.

(3) The pH during the fermentation was also followed with time.

Analysis of the culture filtrates was by hplc - assay conditions were as described before. Pseudomonic acid is produced from approximately 20h and fermentation is essentially complete after 48h. During the fermentation, as the glucose is used up, the pH drops to approximately 4.5 before rising to a finishing pH 0f 8.0.

LABELLED PRECURSOR INCORPORATION STUDIES

All intial feeding studies, until indicated in the text, were carried out using the NCIB 10586 strain of *P. fluorescens* grown using fermentation media 2 and the intial maintenance and culture fermentation conditions outlined previously. The flasks were harvested and isolated pseudomonic acid was methylated and purified as described before. The control flasks for the following incorporation experiments, were monitored by hplc using the assay conditions outlined before.

INCORPORATION OF SODIUM [1-^{14}C]ACETATE

In 3 separate experiments, sodium [1-^{14}C]acetate as a

solution in distilled water (5ml) was filter-sterilised and added to a growing second stage culture flasks of *P. fluorescens* at set time intervals, (detailed below). Each experiment used ten second stage flasks 0.5ml (200mg, 0.5μCi in 5ml of sterile water) of acetate solution was added to each flask. At the same time a set, ten second stage flasks, were grown up as controls and the production of the metabolites monitored by hplc. After 60h the flasks were harvested as detailed previously. Yields of the isolated methyl pseudomonate from the three incorporation experiments are given below. The specific activity of each sample of the isolated methyl pseudomonate was determined by liquid scintillation counting. The results are summarised below.

FERMENTATION TIME (h)	YIELD (mg)	SPECIFIC ACTIVITY (dpm/mmol)	DILUTION PER LABELLED SITE
10	30	6.44×10^4	848
15	20	2.40×10^5	22.7
20	15	1.94×10^6	0.28
CONTROL	50	———	———

INCORPORATIONS OF SODIUM [1-^{13}C,$^{2}H_3$]ACETATE AND [1-^{13}C,$^{18}O_2$]ACETATE

In two separate experiments, 200mg of each of the labelled acetates were fed after 20h fermentation to ten second stage flasks. The fermentation conditions are as detailed before. The flasks were harvested after 48h, again as detailed previously, to yield methyl pseudomonate as a colourless

oil. The [1-^{13}C,$^{2}H_3$]acetate feed yielded 25mg and the [1-^{13}C, $^{18}O_2$]acetate feed yielded 19mg.

MEASUREMENT OF OXYGEN UPTAKE BY *P. FLUORESCENS* AND $^{18}O_2$ INCORPORATION

Ten second stage flasks were set up using the conditions outlined and connected to the $^{18}O_2$ "closed" system apparatus (figure 2.7) by means of flexible tubing. The oxygen uptake by the culture was measured with time, giving the results detailed below. The flasks were harvested after 74h fermentation time and after purification yielded 110mg of methyl pseudomonate as a colourless oil. ^{1}H nmr spectrum was identical to one determined from an authentic sample of methyl pseudomonate.

FERMENTATION TIME (h)	$^{16}O_2$ UPTAKE (cm^3)
1	1071
5	1197
26	2519
28	2796
31	3250
33	3779
50	5063
54	5403
57	5844
74	6386

Total oxygen uptake was 5.3 litres.

For the $^{18}O_2$ incorporation experiment, five second stage flasks were used and were connected to the $^{18}O_2$ apparatus after 24h growth, in time for the phase of maximum oxygen uptake by the culture (this oxygen uptake with time is

detailed below). The flasks were harvested after 44h growth, after which time oxygen usage by the culture had levelled off. This yield 8mg of a white solid. The ^{1}H nmr spectrum of this isolated material was identical to one determined from an authentic sample of methyl pseudomonate.

FERMENTATION TIME (h)	$^{18}O_2$ UPTAKE (cm^3)
24.5	554
26.5	743
28.0	894
29.5	1146
30.5	1265
31.0	1328
43.5	2600
44.0	2650

The total ^{18}Oxygen uptake was 2.1 litres.

SYNTHESIS OF β-HYDROXY-β-METHYL GLUTARIC ACID

Sodium acetate (112), (1g, 12mmol) was mixed with tri-^{n}Butyl phosphate (4ml) and heated under reflux for 2h at 200-220°C. The resulting viscous mixture was cooled to room temperature and the upper end of the reflux condenser was sealed through a line containing two traps to a vacuum pump and the product ester was distilled by heating the reaction flask to 160°C for 2.5h. ^{n}Butyl acetate (114) was collected in the second trap which was cooled by liquid N_2. Any tri-^{n}Butyl phosphate which distilled was collected in the first trap which was cooled by an ice/salt bath.

δ_H ($CDCl_3$, 80MHz) 0.9 (3H, t, J=6Hz, $C\underline{H}_3$), 1.5 (4H, m, J=6Hz, 2x$C\underline{H}_2$), 2.0 (3H, s, $C\underline{H}_3$), 4.0 (2H, t, J=6Hz, $OC\underline{H}_2$).

^{n}Butyl acetate (113) (1.23g, 10.6mmol) and allyl bromide (3.85g, 2.7ml, 31.8ml) in a 3/7, v/v mixture of anhydrous diethyl ether/dry THF (50ml) was added to dry magnesium turnings (1.0g, 42.4mmol) in 10ml of the diethyl ether/THF solvent, at such a rate that a moderate reflux was maintained. After the addition was complete, the reaction mixture was refluxed for 1h at 60°C and then stirred at room temperature for 5h. Ice (approximately 100g) was added to the reaction flask which was also cooled in an ice bath before being acidified with 6M H_2SO_4. The acid solution was stirred at room temperature overnight and then extracted with ether (3x 50ml). The combined ethereal extracts were washed with saturated $NaHCO_3$ solution, water, brine and then dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* and the crude product distilled under reduced pressure to yield 4-hydroxy-4-methyl-hepta-1,6-diene (114) (1.57g, 12.4mmol) as a colourless oil. (Bp. 75-80°C, water pump pressure).

δH ($CDCl_3$, 80MHz) 1.18 (3H, s, $C\underline{H}_3$), 1.8 (1H, s, exchangeable O$\underline{H}$), 2.23 (4H, d, J=7Hz, 2x$C\underline{H}_2$), 5.0 - 6.0 (6H, m, vinyl protons)

The diene (114), (1.57g, 12.4mmol) was taken up in 30ml of a 10:1, v/v solution of dichloromethane/acetic acid and cooled to -78°C before being subjected to ozonlysis for 1.5h. The resulting solution was left to stand at room temperature overnight. The solvent was carefully removed *in vacuo* at 30°C. To the resulting colourless gum was added acetic acid (30ml) and hydrogen peroxide (16ml, 50 vol). This mixture was heated under reflux for 24h. The yellow solution was

then cooled to room temperature and the solvent evaporated *in vacuo*. The resulting semi-solid residue which formed on standing at room temperature was recrystallised from acetone/chloroform to yield 3-hydroxy-3-methylpentan-1,5-dioic acid (β-hydroxy-β-methyl glutaric acid) (115), (1.2g, 7.4mmol). Mp = 108-110°C (lit[29(a)] 110-111°C)

δ_H (d_6 acetone, 80MHz) 1.38 (3H, s, $C\underline{H}_3$), 2.68 (4H, s, $2xC\underline{H}_2$), 6.15 (ca.2H, br s, $2xCO_2\underline{H}$)

A solution of (115) (0.6g, 3.7mmol) was converted to the diethyl ester by treatment with a large excess of HCl saturated ethanol solution. This was stirred at room temperature for 24h. The solvent was evaporated *in vacuo* and the residue taken up in ethyl acetate and washed with water (3x 20ml). The ethyl acetate extract was dried over anhydrous $MgSO_4$ and the solvent evaporated *in vacuo* to yield the diethyl ester (117) (0.65g, 2.98mmol, 81%) as an oil.

δ_H ($CDCl_3$, 60MHz) 1.0 (3H, t, J=6Hz, $C\underline{H}_3$), 1.2 (3H, s, $C\underline{H}_3$), 2.7 (4H, s, $2xC\underline{H}_2$), 4.0-4.5 (4H, q, J=6Hz, $2xOC\underline{H}_2$)

The diacid (115), 0.6g (3.7mmol) was taken up in 5ml of water and converted to the di-sodium salt by neutralisation with 2M NaOH (3.7ml). This solution was stirred at room temperature overnight and then freeze-dried to yield the di-sodium salt (116) 0.72g (3.6mmol, 97%) as a white solid.

δ_H(D_2O, 80MHz) 1.3 (3H, s, $C\underline{H}_3$), 2.4 (4H, s, $2xC\underline{H}_2$).

SYNTHESIS OF [3-^{14}C]-β-HYDROXY-β-METHYLGLUTARIC ACID

This radio-labelled ester was prepared as described above for the unlabelled synthesis. Sodium acetate 1g (specific activity 125μCi/g) was reacted with tri-^{n}butyl phosphate (4ml), to give the labelled ^{n}butyl acetate (1.23g, 10.6mmol, 88%). This was then reacted with allyl bromide (3.85g, 2.7ml, 31.8mmol) in 15ml of a 3/7 v/v mixture of anhydrous diethyl ether/THF and magnesium (1g, 42.4 mmol). This product was then used directly in the ozonolysis reaction and subsequent oxidative workup to yield [3-^{14}C]-β-hydroxy-β-methylglutaric acid (800mg, 5.1mmol). This was re-crystallised from acetone/chloroform to yield 600mg (3.8mmol, 36% overall from ^{n}butyl acetate). The specific activity of this compound as determined by liquid scintillation counting was 9μCi (1.09×10^{7} dpm/mmol).

This labelled glutaric acid (202mg, 1.3mmol), was converted to the diethyl ester by reaction with an excess of HCl saturated ethanol. The solution was stirred at room temoperature for 24h and the solvent was evaporated *in vacuo*. The residue was taken up in ethyl acetate and washed with water (3x 10ml), dried over anhydrous $MgSO_4$ and the solvent removed *in vacuo* to yield the diethyl ester (250mg, 1.2mmol, 92%) as a brown oil.

[3-^{14}C]-β-hydroxy-β-methylglutaric acid, (400mg, 2.6mmol), was converted to the di-sodium salt by the method outlined previously for the synthesis of the unlabelled compound. This yielded (505mg, 2.5mmol, 96%).

SYNTHESIS SODIUM [3,6-$^{13}C_2$]-β-HYDROXY-β-METHYLGLUTARATE

This was accomplished by the route outlined previously for the unlabelled compound. [1,2-$^{13}C_2$]^{n}Butyl acetate was generated from sodium [1,2-$^{13}C_2$]acetate 1g, (90 atom %) reacted with tri-^{n}butyl phosphate (4ml). This yielded the labelled ^{n}butyl acetate, 1.16g (10.0mmol, 82%).

[3,6-$^{13}C_2$]-4-Hydroxy-4-methyl-hepta-1-6-diene was generated by reacting the labelled ^{n}butyl acetate (1.16g, 10mmol) in a Grignard reaction using allyl bromide (2.56ml, 1.42g, 30mmol) and magnesium (0.96g, 40mmol) with dry diethyl ether/THF (3/7, v/v) as the solvent. This yielded the labelled diene, 1.55g (12.3mmol), as a brown oil which was used directly in the ozonolysis reaction to form [3,6-$^{13}C_2$]-β-hydroxy-β-methyl glutaratic acid after oxidative workup with hydrogen peroxide and glacial acetic acid. The crude product was re-crystallised from acetone/chloroform to yield 0.8g (5.1mmol), as an off white solid.

δC (d_6 acetone, 90.56MHz)	26.2 (q, d pent, $J_{\underline{C}-\underline{H}}$=127Hz, $J_{\underline{C}-\underline{C}}$=40Hz, $J_{C-C-C-H}$=5Hz, C-6)
	69.4 (d oct, J_{CC}=40Hz, J_{CCCH}=5Hz, C-3)
δH (d_6 acetone, 200.13MHz)	1.38 (3H, dd, J_{CH}=121Hz, J_{HCCCH}=5Hz, $C\underline{H}_3$)
	2.68 (4H, dd, J_{HCCCH}=5Hz, 2x$C\underline{H}_2$)
	5.5 (2H, br s, 2x$CO_2\underline{H}$).

This $^{13}C_2$ labelled compound was also converted to the

di-sodium salt, by the method outlined previously for feeding studies. This yielded 0.97g (4.8mmol, 95%).

INCORPORATION OF SODIUM AND DIETHYL [3-^{14}C]-β-HYDROXY-β-METHYLGLUTARATES

In separate experiments;

(1) 250mg of di-sodium [3-^{14}C]-β-hydroxy-β-methyl glutarate as a solution in 10ml sterile water (1ml/flask) and

(2) 250mg of diethyl [3-^{14}C]-β-hydroxy-β-methylglutarate as a solution in 0.5ml ethanol (0.05ml/flask)

were each fed to ten second stage flasks, set up as detailed previously, after 20h fermentation. The specific activity of these precursors was 1.98×10^7dpm/mmol. The flasks were harvested after 48h fermentation. Yields, specific activity of the isolated metabolite and dilution values (assuming 2 labelled sites are detailed below.

PRECURSOR	YIELD mg	SPECIFIC ACTIVITY (dpm/mmol)	DILUTION
(1) Di-sodium salt feed	24	9.32×10^6	4.2
(2) Di-ethyl ester feed	20	2.74×10^6	14.4

INCORPORATION OF DI-SODIUM [3,6-$^{13}C_2$]-β-HYDROXY-β-METHYLGLUTARATE

For this incorporation study, the modified culture conditions were used. Ten second stage culture flasks were set up as described previously. 250mg (as a solution in 10ml sterile water) of this precursor was added to this

growing culture after 20h fermentation (1ml/flask). After 40h fermentation, the flasks were harvested, and worked up as described previously, to yield 43.3mg of a white solid.

SYNTHESIS OF DIETHYL MALONATE

Freshly distilled triethyl phosphate (7.5ml) was added to sodium acetate (2.0g, 24.4mmol) and the mixture refluxed at 170-185°C for 3h. The reaction mixture was cooled and then the upper end of the reflux condenser was sealed to a vacuum line *via* a two trap line. The reaction mixture was then warmed to 120°C (0.6mbar) and the **ethyl acetate (118)** was collected in the second trap which was cooled by liquid N_2. Any triethyl phosphate which distilled was collected in the first trap which was cooled by ice/salt bath. This yielded 1.84g of (118) (21mmol, 86%)

δ_H ($CDCl_3$, 80MHz) 1.2 (3H, t, J=8Hz, $C\underline{H}_3$), 2.0 (3H, s, $C\underline{H}_3$), 4.18 (4H, q, J=8Hz, $C\underline{H}_2$)

^{n}Butyl lithium 17.5ml (as a 1.6M solution in hexanes (28mmol)) was added to a stirred solution of hexamethyldisilane (HMDS) (4.71ml, 3.01g, 22.4mmol) in 30ml dry THF at -70°c under a N_2 atmosphere. The solution was allowed to warm slowly to 0°c and then re-cooled to -70°c. Ethyl acetate, 1.4g (1.5ml, 15.3mmol) was dissolved in 4ml of dry THF and was added dropwise to the reaction mixture and the resulting mixture was stirred for 30min at -70°c. Ethyl chloroformate (1.7g, 1.8ml, 18.4mmol) was then added keeping the temperature below -55°c. After a further 2.5h at -70°c, 6M HCl (4ml) was added along with water (20ml) and

diethyl ether (100ml). The organic and aqueous layers were separated and the aqueous layer further extracted with diethyl ether (50ml). The diethyl ether extracts were combined, washed with 3M HCl (20ml), 5% $NaHCO_3$ (50ml) and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to yield diethyl malonate (119) (2.1g, 13.1mmol, 86%).

δH ($CDCl_3$, 80MHz) 1.25 (6H, t, J=6Hz, $C\underline{H}_3$), 3.32 (2H, s, $C\underline{H}_2$) 4.18 (4H, q, J=6Hz, $C\underline{H}_2$)

SYNTHESIS OF DI-SODIUM MALONATE

Conversion of this diethyl ester to the di-sodium salt was achieved as follows; 250mg (1.56mmol) of diethyl malonate was taken up in 5ml of water and titrated with 0.1M NaOH solution using an automatic titrating apparatus. The starting pH was 11.80 and as hydrolysis proceeded it was maintained by automatic addition of the NaOH solution. The volume of NaOH taken up during the hydrolysis was 28.89ml (theoretical amount required was 31.6ml). The aqueous solution was washed with n-hexane (3x 10ml) and then the hexane extracts were combined and back extracted with water (20ml). The aqueous extracts were combined and freeze-dried to yield di-sodium malonate (120) (186mg ,1.45mmol, 93%) as a white solid.

υ_{max} (KBr) $1244cm^{-1}$, $1387cm^{-1}$ C-O stretches, $1605cm^{-1}$ enol C=C stretch, $1717cm^{-1}$ carbonyl stretches

δ_H (D_2O, 80MHz) 2.9 (2H, s, $C\underline{H}_2$)

SYNTHESIS OF DIETHYL [2-^{13}C]MALONATE

This labelled compound was prepared as described for the

unlabelled synthesis. Sodium [2-^{13}C]acetate, 2g (24.4mmol, 90 atom %) was converted to ethyl acetate by reaction with triethyl phosphate (7.5ml). This yielded ethyl [2-^{13}C]acetate, (1.84g, 21mmol, 86%).

δ_H ($CDCl_3$, 80MHz) 1.2 (3H, t, J=8Hz, $C\underline{H}_3$), 2.0 (2H, d, $J_{C-\underline{H}}$=129Hz, $C\underline{H}_3$), 4.18 (4H, q, J=8Hz, $C\underline{H}_2$)

This labelled ethyl acetate was then converted to diethyl [2-^{13}C]malonate by using HMDS (5.3ml) and ^{n}butyl lithium (23ml), (1.4M solution in n-hexanes) ,in dry THF, to generate the enolate of ethyl acetate which was then condensed at -78^{o}C with ethyl chloroformate (1.45ml, 1.65g, 17.84mmol). This produced diethyl [2-^{13}C]malonate (1.88g, 11.7mmol, 76%).

δ_H ($CDCl_3$, 80MHz) 1.25 (3H, t, J=6Hz, $C\underline{H}_3$), 3.4 (2H,d, $J_{\underline{C}-\underline{H}}$=130Hz, $^{13}C\underline{H}_2$), 4.15 (4H, q, J=6Hz, $C\underline{H}_2$)

This labelled diethyl malonate, (250mg, 1.56mmol), was converted to the corresponding di-sodium salt by automatic titration with 1.0M NaOH solution. The volume of NaOH solution taken up was 2.3ml (theoretical volume required = 3ml). After workup, as described previously, di-sodium [2-^{13}C]malonate as a white solid, (179mg, 1.4mmol, 90%), was obtained.

δ_H (D_2O, 250.13MHz) 3.09 (2H,d $J_{\underline{C}-\underline{H}}$=130Hz, $C\underline{H}_2$)

This synthesis was repeated with more diethyl [2-^{13}C]malonate, (250mg) to give di-sodium [2-^{13}C]malonate (186mg).

This synthesis was again repeated using 1.14g (7.2mmol) of the labelled diethyl malonate. This time, the diethyl malonate was taken up in water (20ml) and 2M NaOH (7.2ml) was added. The resulting solution was stirred at room temperature overnight. The aqueous solution was washed with n-hexane (3x 10ml) and then the hexane extracts were back extracted with water. The aqueous extracts were combined and freeze-dried to yield (7.2mmol) of a white solid. The ^{1}H nmr spectrum was identical to an authentic spectrum.

INCORPORATION OF DIETHYL [2-^{13}C]MALONATE

Diethyl [2-^{13}C]malonate (250mg) was fed as a solution in 1ml of ethanol to ten second stage flasks (0.1ml/flask) after 20h fermentation. The modified fermentation conditions were used for this study. The flasks were harvested after 40h fermentation and 50.5mg of a white solid was isolated.

INCORPORATION OF DI-SODIUM [2-^{13}C]MALONATE

(1) Using the modified culture conditions, ten second stage flasks were set up and after 20h fermentation, di-sodium [2-^{13}C]malonate (250mg) was added to this growing culture. This labelled precursor was fed as a solution in 10ml of sterile water at a level of 1ml/flask. The flasks were harvested after 40h growth and yielded 22.6mg of a white solid.

(2) 1.07g of this labelled precursor was fed as a solution in 10ml of sterile water to five second stage flasks (2ml/flask) after 20h fermentation. The modified

fermentation conditions were used. After 40h growth, the flasks were harvested and 30mg of a white solid was isolated.

INCORPORATION OF SODIUM [^{13}C]BICARBONATE

In this incorporation experiment, the chalk which is used to buffer the second stage of the fermentation, was replaced by ^{13}C labelled bicarbonate. 1g of this labelled bicarbonate was used to make up eight second stage fermentation flasks (*ie.* 0.125g/flask). All other medium constituents were as outlined previously, for the modified culture conditions. The inoculation procedures and fermentation conditions were as described for the modified fermentation conditions. After 40h growth the flasks were harvested. The pH of the culture supernatant was abnormally low (pH 4). Extraction was carried out as described previously, except the pH of the culture supernatant was unaltered. The isolated crude methyl pseudomonate was purified by preparative tlc and a band at rf 3.1 afforded 20mg of a colourless oil which crystallised on standing in the fridge.

INCORPORATION OF SODIUM [1-^{13}C]ACETATE

(1) [1-^{13}C]Acetate (200mg) as a solution in 10ml of sterile water was fed to ten second stage flasks after 20h fermentation (1ml/flask). Again, the modified culture and fermentation conditions were used. After 40h fermentation these flasks were harvested, by the method outlined previously. 40mg of a white solid was

isolated.

(2) [1-^{13}C]Acetate (500mg) was pulse fed to the growing culture. To prevent abnormal changes in the pH during fermentation, the second stage flasks were buffered on the alkaline side by increasing the chalk concentration to 7.5g/l and increasing the amount of Na_2HPO_4 present to 1.5g/l and decreasing the amount of KH_2PO_4 to 1.0g/l. All other constituents were as described previously for the modified culture and fermentation conditions. The [1-^{13}C]acetate was fed in five 100mg portions, in 10ml of sterile water (1ml/flask), after 10, 14, 22, 26, and 30h fermentation. The flasks were harvested after 48h fermentation and yielded, after workup as described previously, 30mg of a white solid.

SYNTHESIS OF SODIUM [1-^{13}C]PROPIONATE

[1-^{13}C]Propionitrile (124) was obtained by reacting potassium [^{13}C]cyanide, (1g, 15.15mmol), with ethyl iodide (2.38g, 1.22ml, 15.25mmol) in 5.0g absolute methanol to which water, 0.24ml, had been added. This suspension was refluxed at 70°C for 12h and then at 80°C for a further 38h. The entire mixture was distilled, and mixed with water, 0.58ml, and 17.88ml of 0.85M potassium ^{t}butoxide in ^{t}butyl alcohol and then refluxed for 48h. The residue obtained upon the evaporation of the solvent was redissolved in distilled water and acidified with phosphoric acid, and then distilled. The distillate was then titrated with NaOH and then lyophilized to generate sodium [1-^{13}C]propionate (122),

0.84g (8.7mmol, 70%).

δ_H ($CDCl_3$, 80MHz) 1.0 (3H, t, $J_{\underline{H}-C-C-\underline{C}}$=5Hz, CH_3), 2.2 (2H, $J_{\underline{H}-C-\underline{C}}$=40Hz, $C\underline{H}_2$)

SYNTHESIS OF 9-HYDROXYNONANOIC ACID

To 8-Bromo-1-octanol (127), (1g, 0.41ml, 2.4mmol) in methanol (20ml) was added sodium cyanide (3.72g), as a solution in water (5ml). The resulting solution was heated under reflux overnight. After cooling the solution, the solvent was evaporated *in vacuo*. The residue was taken up in ethyl acetate (25ml) and washed with water. The ethyl acetate extract was then dried and evaporated to dryness to yield 9-hydroxy-nonanitrile (128) (0.33g, 88%). The water layers were combined and reacted with bleach to destroy any unreacted sodium cyanide which had been extracted into it.

δ_H ($CDCl_3$, 80MHz) 1-2 (12H, br, 6x$C\underline{H}_2$), 2.3 (2H, t, J=5Hz, $C\underline{H}_2$), 3.6 (2H, t, J=5Hz, $C\underline{H}_2$).

This 9-hydroxy-nonanitrile (128) (0.33g, 2.1mmol), was hydrolysed to the corresponding acid by reaction with potassium hydroxide (0.44g, 7.9mmol, 3.8 equiv) in methanol (13ml) and water (5ml). This solution was heated under reflux overnight. After cooling, the solvent was evaporated *in vacuo*. The residue was taken up in a small volume of water and cooled in an ice-bath before the pH was adjusted to 1.0 by addition of 2M HCl. This solution was extracted with ethyl acetate (5x 20ml), dried over anhydrous $MgSO_4$ and the solvent evaporated to yield 9-hydroxynonanoic acid (129), (0.28g, 1.6mmol, 77%).

δ_H ($CDCl_3$, 80MHz) 1-2 (12H, br, 6x$C\underline{H}_2$), 2.2 (2H, t, J=5Hz, $C\underline{H}_2$), 3.6 (2H, t, J=5Hz, $C\underline{H}_2$), 5.0 (2H, br s, 2x$O\underline{H}$.

9-Hydroxynonanoic acid (0.28g, 1.6mmol) was taken up in the minimum volume of methanol and reacted with an excess of diazomethane (generated as described previously). The resulting solution was stirred at room temperature for 1h. Any unreacted diazomethane was removed by bubbling N_2 through the solutiuon. The solvent was evaporated *in vacuo* to yield a waxy solid. This was taken up in diethyl ether (10ml) and filtered. The filtrate was concentrated to yield methyl 9-hydroxynonanoate (132) as a yellow oil (0.15g, 0.8mmol, 50%).

δ_H ($CDCl_3$, 80MHz) 1-2 (12H, br, 6x$C\underline{H}_2$), 2.3 (2H, t, J=5Hz, $C\underline{H}_2$), 3.2 (1H, br s, $O\underline{H}$), 3.6 (2H, t, J=5Hz, $C\underline{H}_2$), 3.7 (3H, s, $C\underline{H}_3$).

Methyl 9-hydroxynonanoate (132), (0.29g, 1.54mmol) was reacted with pyridinium chlorochromate (PCC) (0.5g, 2.31mmol, 1.5 equiv) in dry methylene chloride (10ml) in a conical flask. This solution was stirred at room temperature overnight. The resulting black solution was filtered through a silica plug to remove most of the chromium salts and the filtrate concentrated *in vacuo* to yield a dark green oil. This was further purified by preparative tlc (solvent system, hexane:ethyl acetate, 4:1, v/v) to yield methyl 9-oxononanoate, (133) (0.28g, 1.5mmol, 97%).

δ_H ($CDCl_3$, 80MHz) 1-2 (12H, br, 6x$C\underline{H}_2$), 2.3 (4H, m, 2x$C\underline{H}_2$),

3.7 (3H, s, C$\underline{H}_3$), 9.8 (1H, d, J=5Hz, C$\underline{H}$O).

Methyl 9-oxononanoate, (133) (0.28g, 1.5mmol) was taken up in dry methanol (10ml) in a 50ml round bottomed flask and reduced with sodium borohydride (0.09g, 2.25mmol). This was added slowly to solution with stirring and the resulting mixture was stirred at room temperature for a further 1/2h. The solvent was removed *in vacuo* and ether:water (1:1) was added. The layers were separated and the aqueous layer further extracted with diethyl ether (5x 20ml). The organic extracts were combined, dried and evaporated to yield methyl 9-hydroxynonanoate (134), (0.08g, 0.43mmol, 29%). The combined aqueous layers were continuously extracted with diethyl ether overnight but this failed to improve the yield.

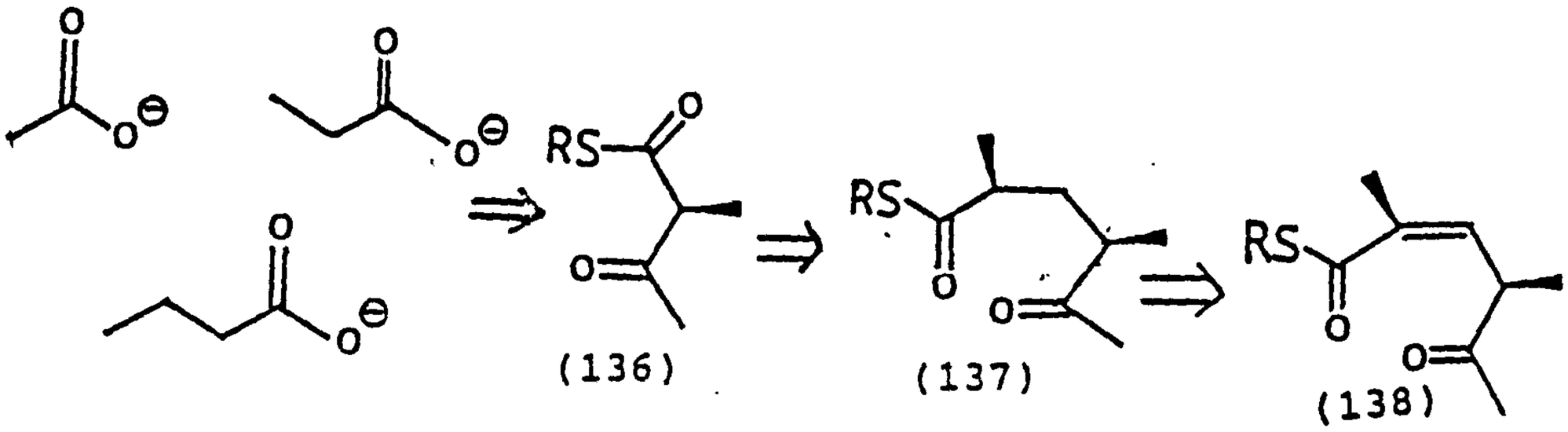
δ_H ($CDCl_3$, 80MHz) 1-2 (12H, br, 6xC$\underline{H}_2$), 2.3 (3H, br m, C$\underline{H}_2$ and O$\underline{H}$), 3.6 (2H, t, J=5Hz C$\underline{H}_2$), 3.7 (3H, s, C$\underline{H}_3$).

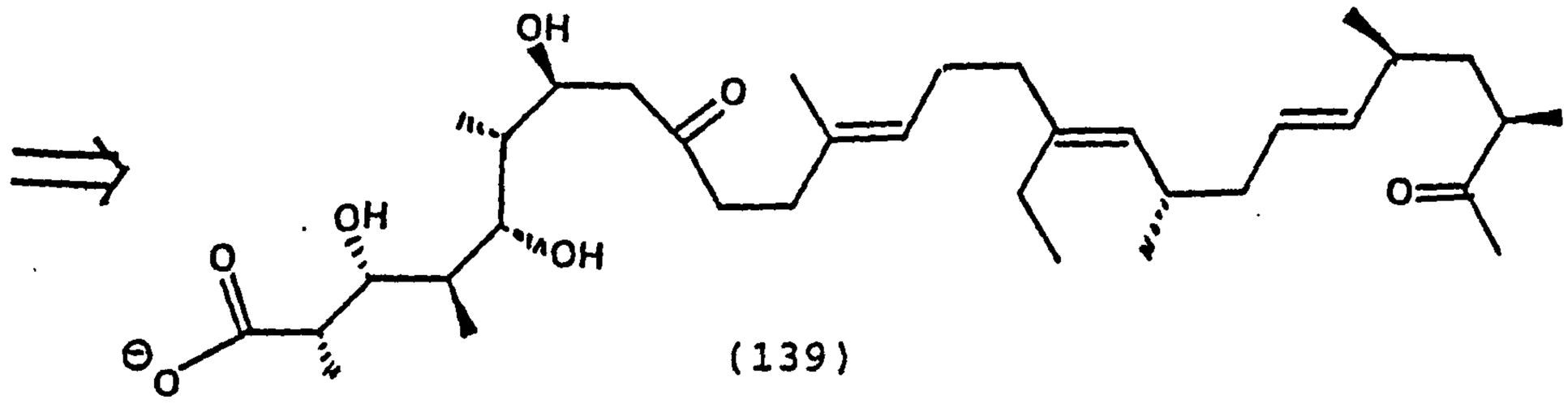
To the methyl 9-Hydroxynonanoate (134), (80mg, 0.43mmol), in water (5ml) was added 2M NaOH (0.12ml). This solution was stirred at room temperature overnight, and then acidified with 2M HCl. This resulting aqueous solution was extracted with diethyl ether (5x 20ml). The organic extracts were combined and dried and evaporated to dryness to give 9-hydroxynonanoic acid (135), (0.06g, 0.35mmol, 81%).

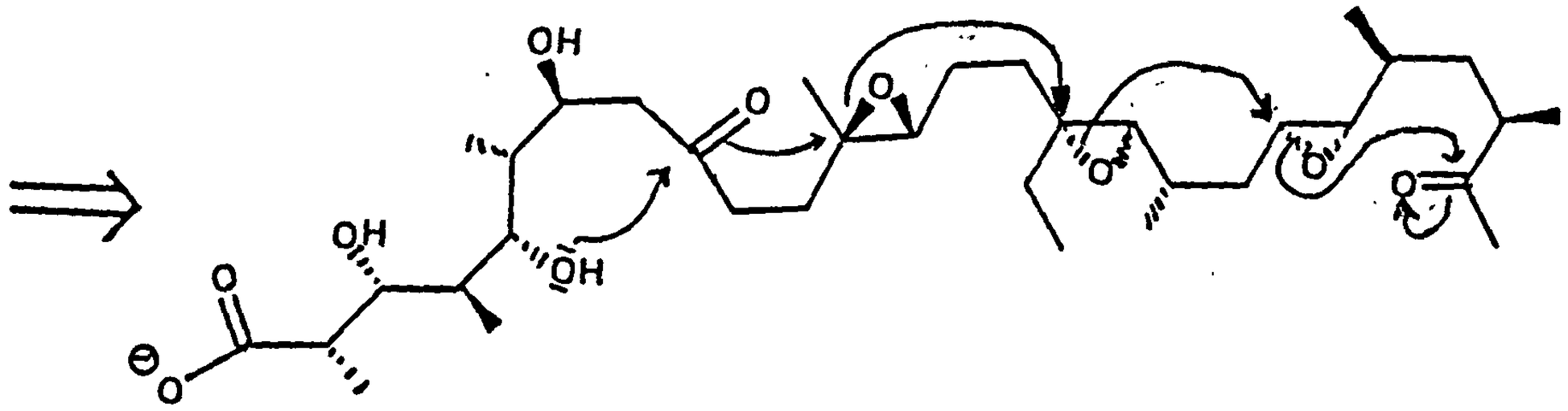
δ_H ($CDCl_3$, 80MHz) 1-2 (12H, br, 6xC$\underline{H}_2$), 2.2 (2H, t, J=5Hz, C$\underline{H}_2$), 3.6 (2H, t, J=5Hz, C$\underline{H}_2$), 5.0 (2H, br s, 2xO$\underline{H}$).

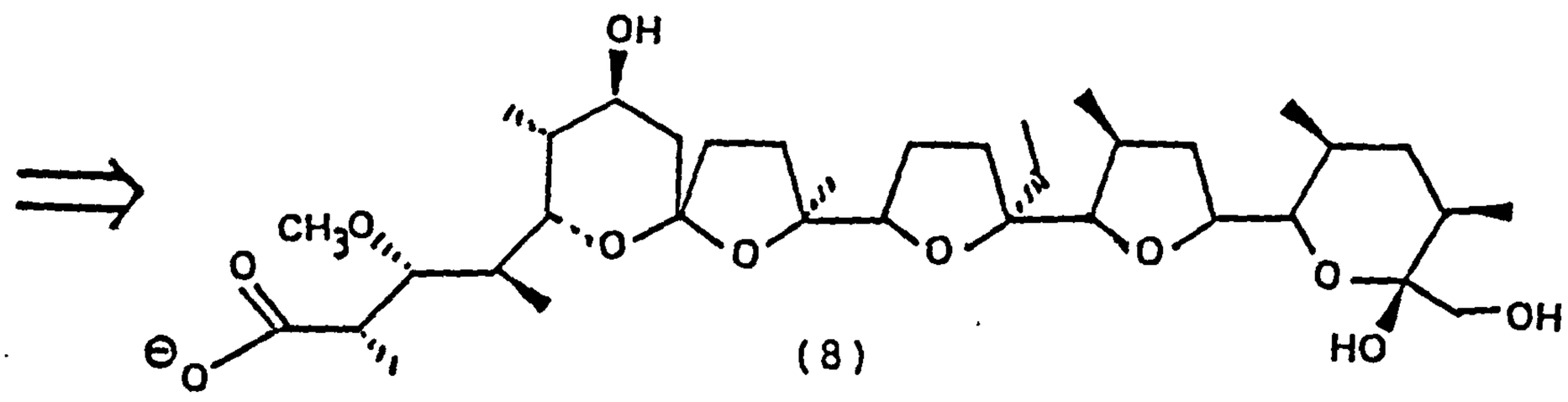
CHAPTER THREE

Synthesis and Incorporation of Advanced Precursors









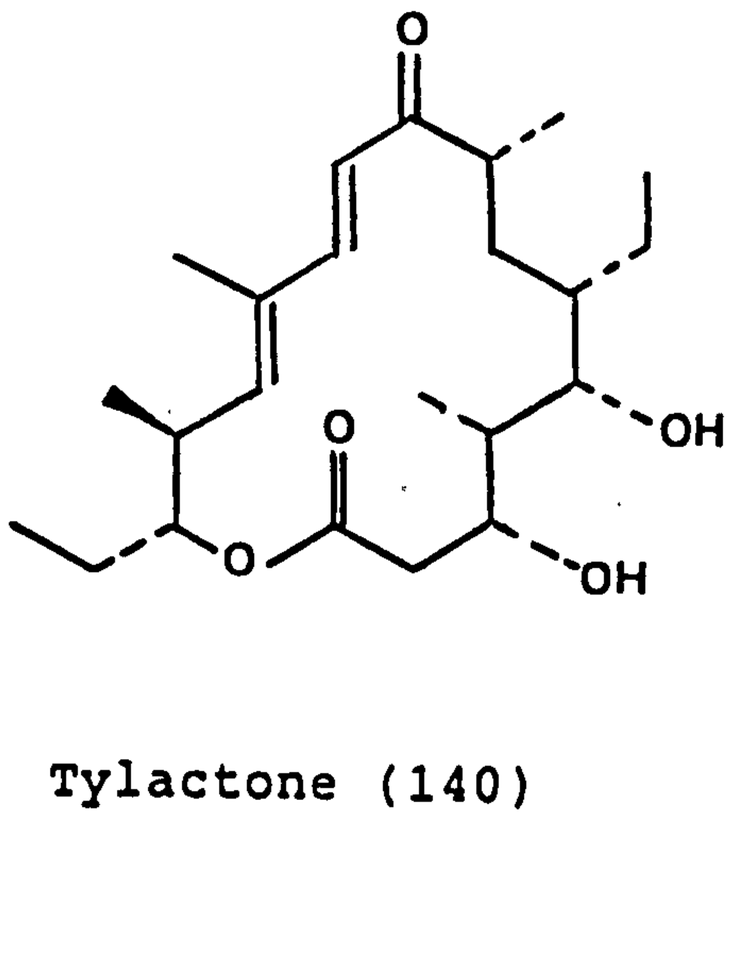
Scheme 3.1

Synthesis and Incorporation of Advanced Precursors

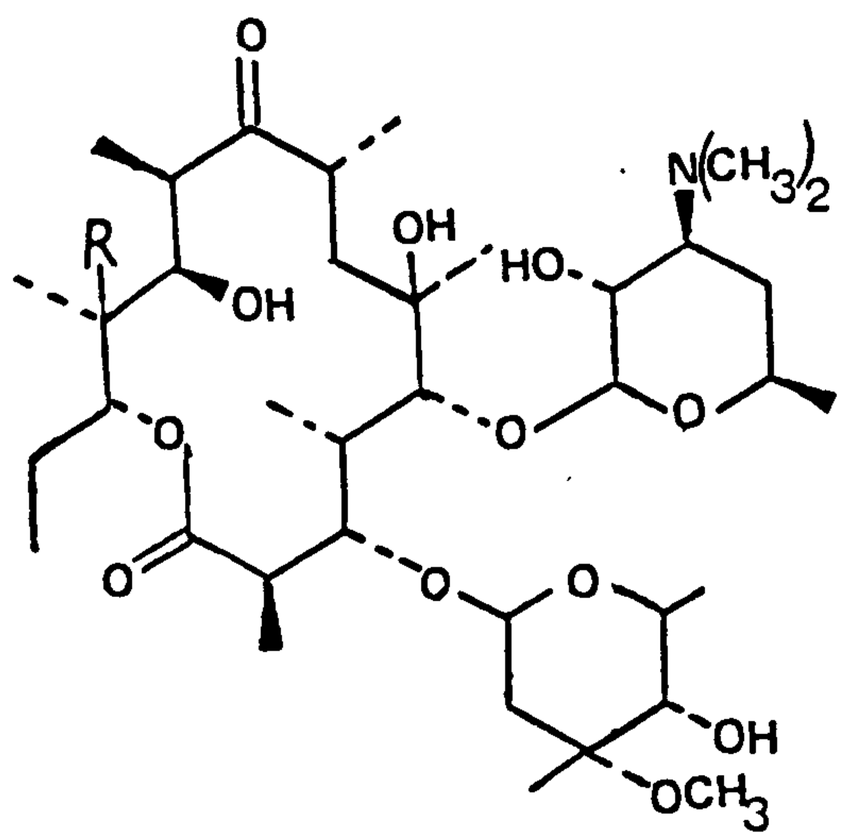
3.1 INTRODUCTION

In 1983, Cane[39] proposed a unified mechanism for the biosynthesis of polyether antibiotics, based on the cascade cyclisation of a postulated polyepoxide intermediate derived from the corresponding polyenes. For example, if the formation of the polyketide chain of monensin (7) were to be analogous to fatty acid biosynthesis, then the necessary units of acetate, propionate, and butyrate should be condensed sequentially, with concurrent adjustment of oxidation and unsaturation, as illustrated in scheme 3.1. Therefore, the intermediate compounds (136) to (138) represent plausible five-carbon, eight-carbon and ten-carbon polyketide chain elongation intermediates, while premonensin (139) corresponds to triene intermediate[41-43] proposed to be the product of the polyketide synthetase enzymes.

Previous attempts to study the incorporation of chain assembly intermediates in whole cell cultures have usually been frustrated by the breakdown of the precursors by catabolic enzymes before they could be incorporated intact into the final metabolite. However, recently it has been found that thioester derivatives of presumed chain elongation intermediates can be incorporated, into macrolide antibiotics, in whole cell studies without prior degradation. For example, in tylactone (140) biosynthesis[44], using cultures of *Steptomyces fradiae*, both a six-carbon propionate-propionate and a nine-carbon tri-propionate

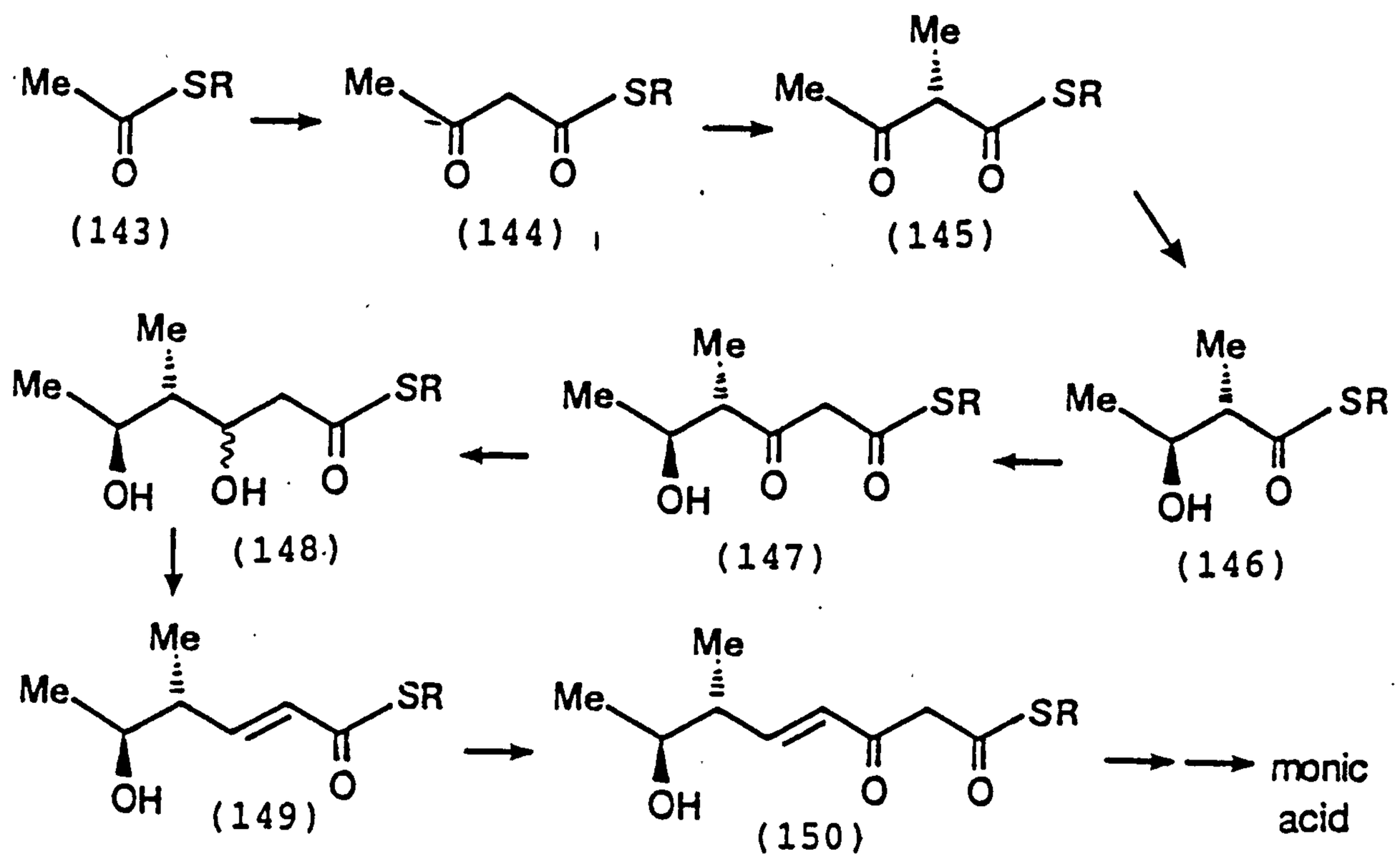


Tylactone (140)



Erythromycin A (141) R= OH

Erythromycin B (142) R= H



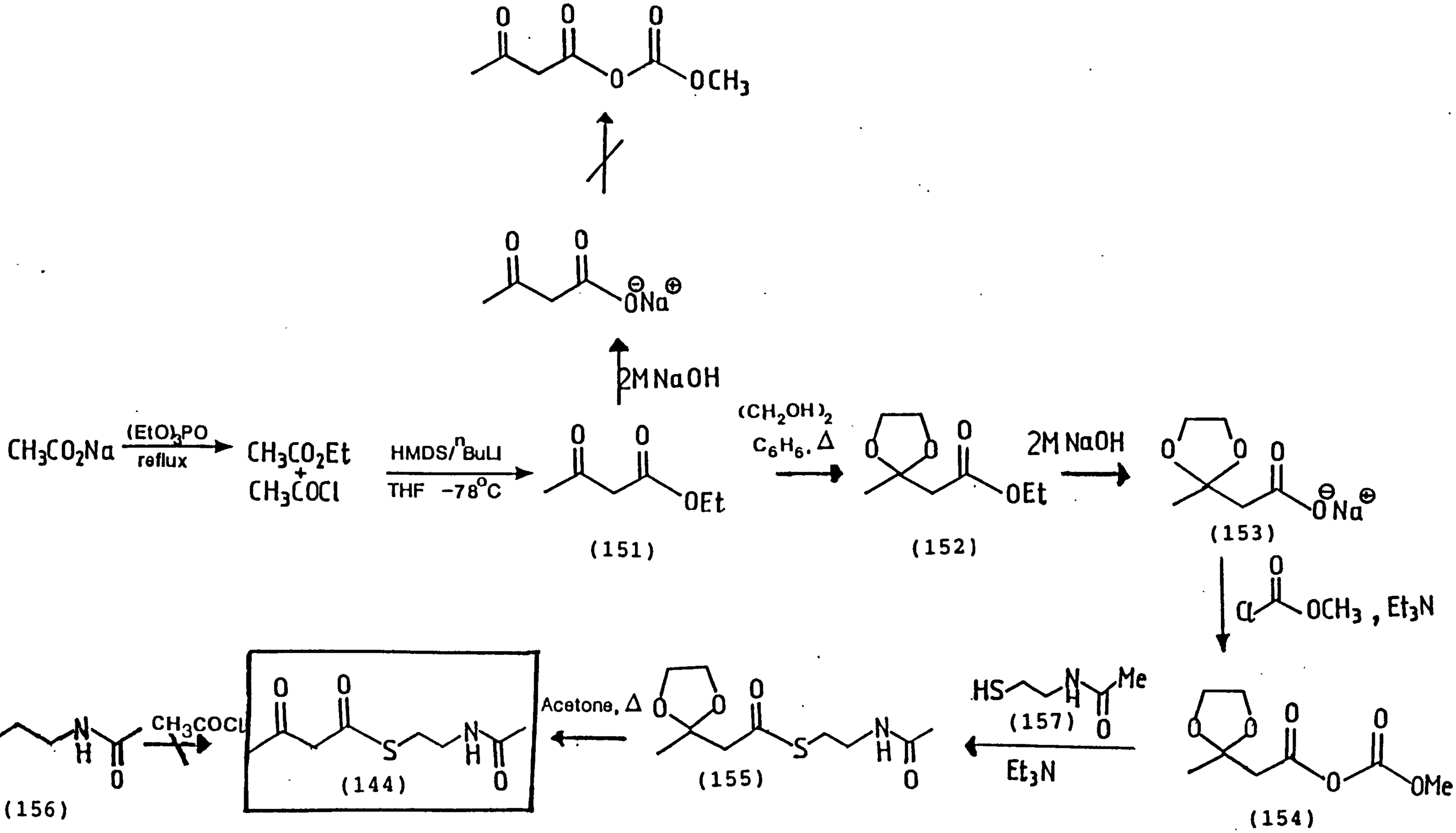
Scheme 3.2

precursor have been incorporated as the corresponding N-acetylcysteamine (NAC) thioesters into the metabolite. Similarly, the NAC thioester of a six-carbon propionate-propionate intermediate has been incorporated into the erythromycins A and B (141) and (142) by *Saccharopolyspora erthraea*[45].

This is stepwise polyketide chain assembly and differs from fatty acid biosynthesis in that it characteristically does not involve the systematic repetition of the same multi-step process in extending the carbon chain. Instead, each time carbon chain extension occurs, the resulting β-keto thioester intermediate undergoes differing degrees of modification, for example, by reduction, dehydration, methylation or oxidation, before the next chain-extending reaction.

3.2 A STEPWISE ASSEMBLY MECHANISM TO MONIC ACID

For pseudomonic acid biosynthesis, a similar stepwise assembly of chain elongation intermediates can be proposed (scheme 3.2). According to this mechanism, enzyme bound acetyl CoA would condense with malonyl CoA to generate enzyme bound acetoacetate (144), which is then stereospecifically methylated to intermediate (145). The β-ketone group is then reduced to generate enzyme bound (2S,3S)-3-hydroxy-2-methylbutanoic acid (146) or alternatively, both stereocentres may be generated in the reduction step. This intermediate (146) is further chain extended by a two-carbon unit from acetyl CoA to generate


CH3CO2Na
(EtO)3PO
reflux
CH3CO2Et
CH3COCl
HMDS/nBuLI
THF −78°C
(151)
(CH2OH)2
C6H6, Δ
(152)
2M NaOH
(153)
Cl
OCH3 , Et3N
(154)
HS
N
H
Me
(157)
Et3N
Acetone, Δ
(155)
(144)
CH3COCl
(156)
2M NaOH

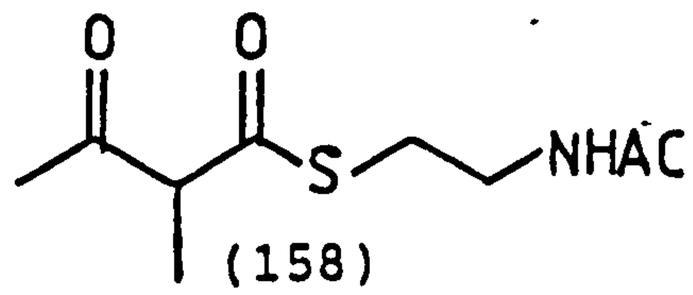
Scheme 3.3

intermediate (147), which is again modified by ketone group reduction to an alcohol, followed by dehydration to generate intermediate (149). This is further chain extended and modified in a stepwise assembly to eventually produce the intermediate precursor to monic acid (2).

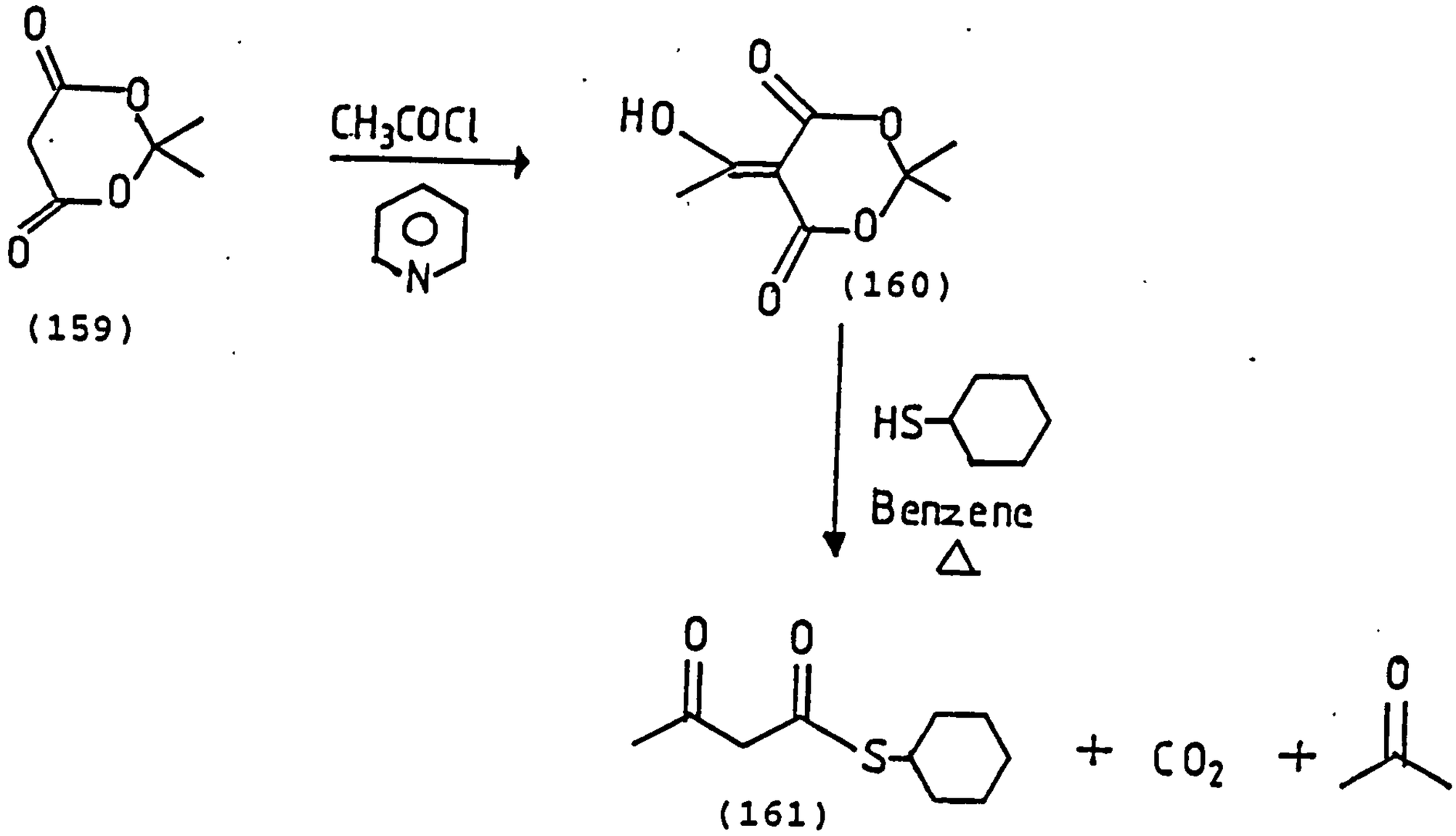
To test this stepwise assembly mechanism, it was decided to examine the incorporation of some of these proposed assembly intermediates. These chain-elongation intermediate compounds had to synthesised in labelled form with the correct stereochemistry. The compounds chosen for study were the acetoacetate intermediate (144), the (2S,3S)-3-hydroxy-2-methylbutanoic acid intermediate (146) and the (4S,5S,2E)-5-hydroxy-4-methylhex-2-enoic acid intermediate (149). These compounds were all synthesised in the following three forms; (i) NAC thioesters, (ii) ethyl esters and (iii) sodium salts. This allows for comparison incorporation of the precursor by the cells into the final metabolite, whether intact or not.

3.3 SYNTHESIS OF PROPOSED CHAIN-ELONGATION INTERMEDIATES OF PSEUDOMONIC ACID BIOSYNTHETIC PATHWAY

The N-acetylcysteamine deriviative of acetoacetate (144), was synthesised as shown in scheme 3.3. Ethyl acetoacetate (151) was synthesised from acetyl chloride and ethyl acetate in a low temperature condensation reaction, using HMDS and ^{n}butyl lithium to generate the enolate of ethyl acetate. Although, the ester (151) could be readily hydrolysed with aqueous alkali, there were the problems due to the acidity



(158)



(159) (160) (161)

Scheme 3.4

of the α-hydrogens and the free acid was also very susceptible to decarboxylation and all attempts to form the thioester (144) from the free acid using standard conditions were unsuccessful.

An alternative approach, to this compound (144) was tried. The N,S-diacetylcysteamine was readily synthesised from 2-mercaptoethylamine hydrochloride by Schwab and Klassen's procedure[50]. The dilithio deriviative of N,S-diacetylcysteamine (156), which was generated by reaction with LDA, was quenched with acetyl chloride. This should have led directly to the desired compound (144). However, this approach was unsuccessful with no trace of the thioester being formed, although a similar NAC thioester of 2-methyl-3-oxobutanoic acid (158) has been prepared recently[51] using this approach. Another route to (144) was developed based on the Yonemitsu method for β-ketoesters using Meldrum's acid (159) as the starting material[52,53,54]. This route is shown in scheme 3.4. In this synthesis, the first step is to acetylate Meldrum's acid. A model reaction using a simpler thiol, cyclohexane thiol, was tried to see if the thiolysis reaction would work. This did give the cyclohexane thiol derivative of acetoacetate (161). However, when the reaction was repeated using N-acetylcysteamine, the reaction was unsuccessful with free thiol being isolated as the main product. Increasing the reaction time also failed to produce the desired product (144).

It was decided to re-examine the first route, scheme 3.3, to

the desired thioester (144). It was thought that protection of the β-ketone group as a ketal[47] might solve both problems of the acidity of the α-hydrogens and the unstability of the free acid to decarboxylation. This was achieved by reacting ethyl acetoacetate with ethylene glycol in refluxing benzene using a Dean-Stark apparatus, to yield ethyl acetoacetate ethylene ketal (152) in 75% yield. Hydrolysis of this ester with base went smoothly and this was then activated as a mixed anhydride (154) by reaction with methyl chloroformate for coupling to N-acetylcysteamine. This proceedure was adapted from thioester preparations described by Hutchinson[46]. This did result in the protected form of the desired thioester (155), in good yield (71%). Deprotection was achieved *via* a trans-ketalisation reaction using acetone and toluene-4-sulphonic acid. However, the reaction time was found to be critical with 2.5h being optimum before the thioester starts to decompose. The thioester was purified by flash chromatography. Initially, the running solvent was unbuffered but free thiol (157) was isolated and ^1H nmr spectrum of the crude product did not show this to be present intially, as there was no triplet signal at δ1.5 characteristic of the HS group. To counteract the slight acidity of the silica, the running solvent was buffered with a trace of triethylamine and this solved the problem of cleavage of the thioester group.

All intermediate compounds were analysed by ^1H nmr spectroscopy. The ^1H nmr spectrum of ethyl acetoacetate (151) showed keto-enol tautomerism. Approximately 5% enol form exists at room temperature of ethyl acetoacetate and

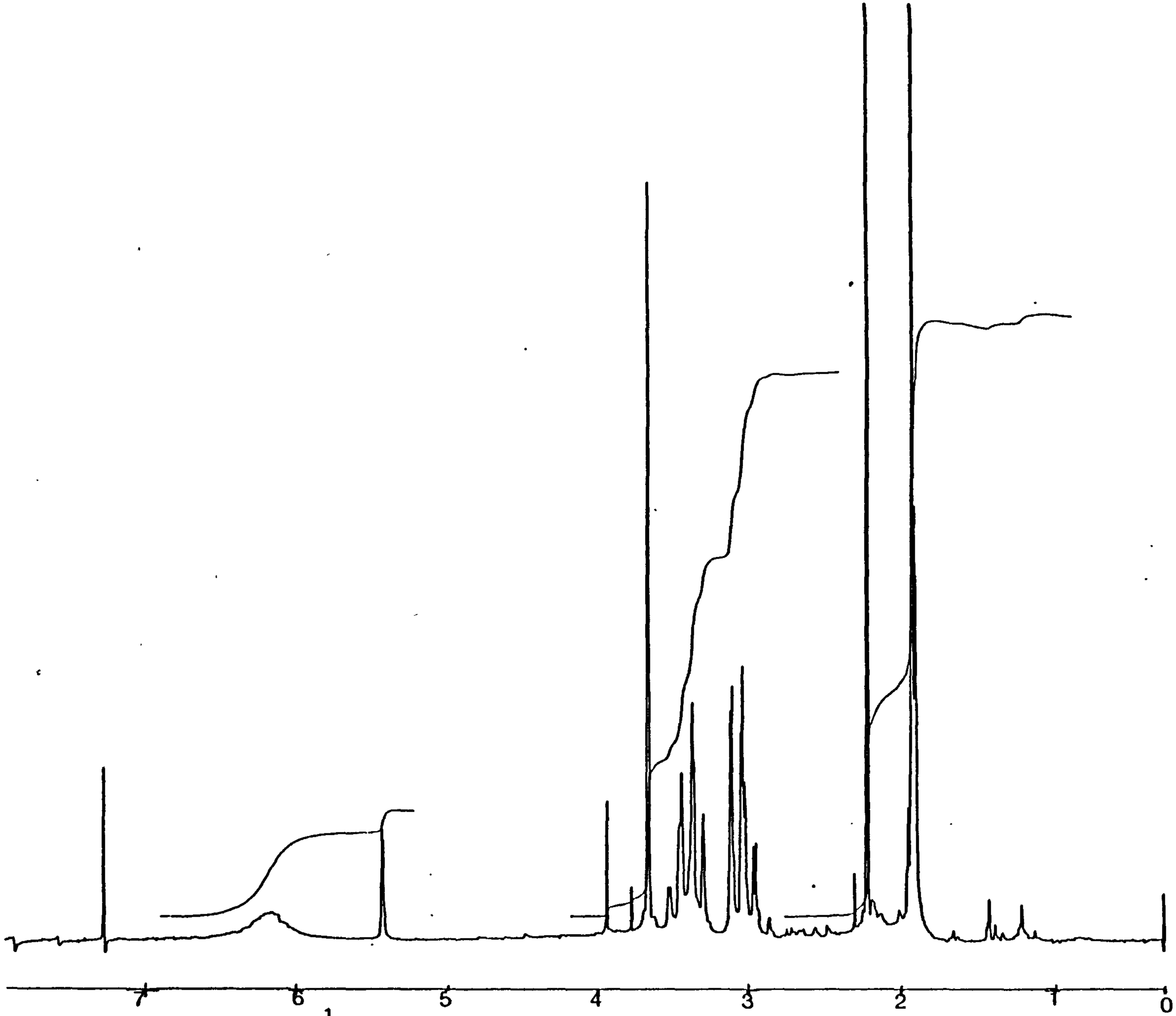


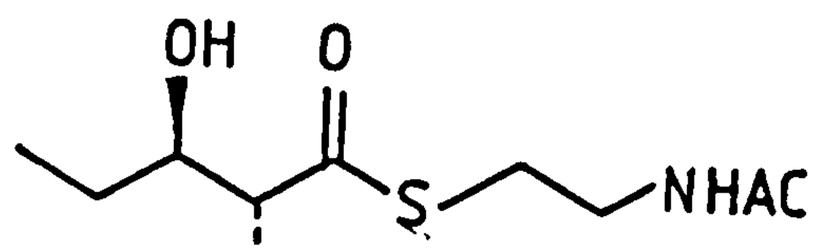
Figure 3.1 ^{1}H nmr spectrum of NAC thioester of acetoacetate.

(170)
OH
O
ONa
N3PO(OPh)2
(165)
(164)
OEt
yeast
reduction
(162)
CH3I, LDA
-78°
CO2Et
(163)
CF3CO2H
CH2Cl2
OTHP
Cl
OCH3
Et3N THF
(168)
(167)
2M NaOH
MeOH
(166)
HS
N
H
CH3
, Et3N
THF
(169)
CF3CO2H
MeOH
S

Scheme 3.5

this is easily seen in the ^{1}H nmr spectrum. The keto-enol spectrum consists of signals from both tautomers. The major signals due to the keto form are easily assigned, and those of the enol tautomer are the singlet methyl signal at δ1.9 and the olefinic proton at ca. δ5.1. The ethyl ester signals coincide for both tautomers. The final thioester (144) also shows similar keto-enol tautomerism. The ^{1}H nmr spectrum (figure 3.1) of this compound also showed characteristic thioester signals at δ3.0 ($C\underline{H}_2S$) and δ3.4 ($C\underline{H}_2NH$). In the free thiol, these signals are at δ2.7 ($C\underline{H}_2SH$) and δ3.4 ($C\underline{H}_2NH$). Mass spectrometry gave a molecular ion at m/z 204 (M^+) as further proof that this thioester had been sucessfully synthesised. This synthetic route allows for the introduction of a double ^{13}C label spanning the acetate-malonate junction, by synthesising the ethyl acetoacetate from ethyl [2-^{13}C]acetate, generated from sodium [2-^{13}C]acetate[31], and [1-^{13}C]acetyl chloride.

Intially, the synthesis of racemic NAC thioester of 3-hydroxy-2-methylbutanoic acid intermediate (170), was based on the general selective procedure by Masamune[55], shown in scheme 3.5. The first step in this synthesis was methylation of racemic ethyl 3-hydroxybutyrate using Seebach's method[49] to generate ethyl 3-hydroxy-2-methylbutyrate, (163), which was then hydrolysed to the free acid using base in aqueous methanol. However, this hydroxy acid proved to be very water soluble and continuous extraction of the aqueous phase into diethyl ether was required to obtain a reasonable yield. This is a disadvantage as this route was to be used to synthesise the



(171)

optically active NAC thioester of (2S,3S)-3hydroxy-2-methylbutanoic acid, (170), and racemisation of the product might occur during this prolonged extraction. Work on this route was continued to the final thioester, by reacting the hydroxy acid with N-acetylcysteamine, using diphenyl phosphorane (DPPA) (165) as the coupling agent[58]. The required thioester (170) was formed, but the ^{1}H nmr spectrum revealed that the coupling agent had also reacted with the hydroxyl group on C-3, indicated by the presence of phenyl resonances. Thin layer chromatography revealed that this was predominately one compound and not a mixture of a by-product from the coupling agent and the desired thioester (170). An alternative approach, to a similar NAC thioester, ie. N-acetylcysteamine thioester of (2R,3R)-3hydroxy-2-methylpentanoate (171), was described recently[44] and this was applied to the coupling of the NAC thioester to the sodium salt of the THP protected ethyl 3-hydroxy-2-methylbutyrate (167), to try to generate the desired thioester (170). The approach is shown in scheme 3.5, and was first tried using racemic ethyl 3-hydroxybutyrate as the starting material. As in the previous synthesis, the first step was a methylation reaction on ethyl 3-hydroxybutyrate, using Seebach's method[49], and then the hydroxy group on C-3 was protected as the THP ether before the ester group was hydrolysed using base. Coupling to give the N-acetylcystamine thioester was achieved by activating the sodium salt of the acid (167) as the mixed anhydride using methyl chloroformate, followed by reaction with N-acetylcystamine, and then deprotection, using a catalytic amount of trifluoroacetic acid in

methanol. These mild reaction conditions ensured that the acid sensitve NAC thioester group was not lost during this reaction. This route did produce the desired thioester (170) in reasonable yield. Proof that this synthesis was sucessful came from ^{1}H nmr analysis and high resolution mass spectrometry which gave a molecular ion at 220 as expected. This route was subsequently used to generate the optically active form starting from ethyl (3S)-3-hydroxybutyrate, as detailed below.

The optical purity of the thioester of (2S,3S)-3-hydroxy-2-methylbutanoic acid (170), which is not a literature compound, was determined by ^{1}H nmr using a chiral solvating agent. These CSA agents are diamagnetic and are used to dissimarily perturb the nmr spectra of enantiomeric solutes. The CSA and the solute must have complementary functionality which permits their interaction *ie.* in general, one is a hydrogen bond acceptor and the other is a hydrogen bond donor. The CSA used in this experiment was R-(-)-trifluoroanthryl ethanol (TFEA). This attractively interacts with the enantiomeric solute (the NAC thioester), resulting in different spacial enviroments for their nuclei. Non-equivalence increases with an increase in CSA concentration until the solute is completely solvated by the CSA. Non-equivalence arises under these circumstances only from spectral differences in the diastereomeric solvates, not from different degrees of association of the solute enantiomers with the CSA, since no diminution of non-equivalence is observed even at high CSA concentrations. In enantiomeric excess (ee), it is necessary to determine

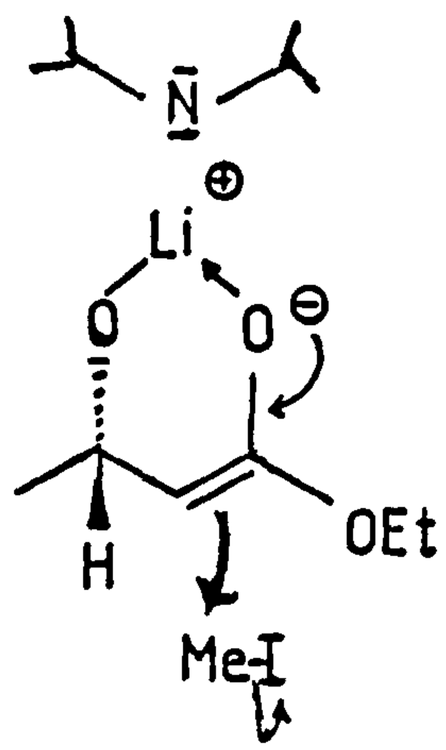


Figure 3.2 A mechanism for the stereospecific methylation reaction.

that the signals being compared truly represent the enantiomeric ratio. Strictly, areas under peaks should be compared in making the ee determinations. However, in practice, it is sufficient to compare peak heights of the diastereotopic resonances. To optimise conditions for determining the resonances where non-equivalence may be readily observed, an intial experiment on racemic material is used. This whole method for determining the enantiomeric purity of compounds is well reviewed by Pirkle and Hoover[61].

The first step in this chiral synthesis of (170) was a stereospecfic baker's yeast reduction[48] of ethyl acetoacetate. This reaction was carried out intially using fresh baker's yeast (*Saccharomyces cerevisiae*). It was subsequently repeated using DCL active dried yeast (single strain, *Saccharomyces cerevisiae*). Both proceedures produced ethyl (3S)-3-hydroxybutyrate in a yield of 48-50%. The optical purity of the products were measured by determining its optical rotation and comparing these to the literature value[48(a)] of +38.5. , $[\alpha]_D^{20}$ = +36.5° for fresh yeast reaction product (% optical purity = 95%), and $[\alpha]_D^{20}$ = +36.1° for the dried yeast product (% optical purity = 94%), Ethyl (3S)-3-hydroxybutyrate (162) was then stereospecifically methylated[46,49]. In this reaction, this lithium base blocks the top face of (162) by complexing between the hydroxyl group and the ester carbonyl, so forcing methylation to occur on the lower face (figure 3.2), giving (163) in a yield of 66%. The optical purity of the ethyl (2S,3S)-3-hydroxy-2-methylbutyrate (163) produced was determined from optical rotation measurements. The

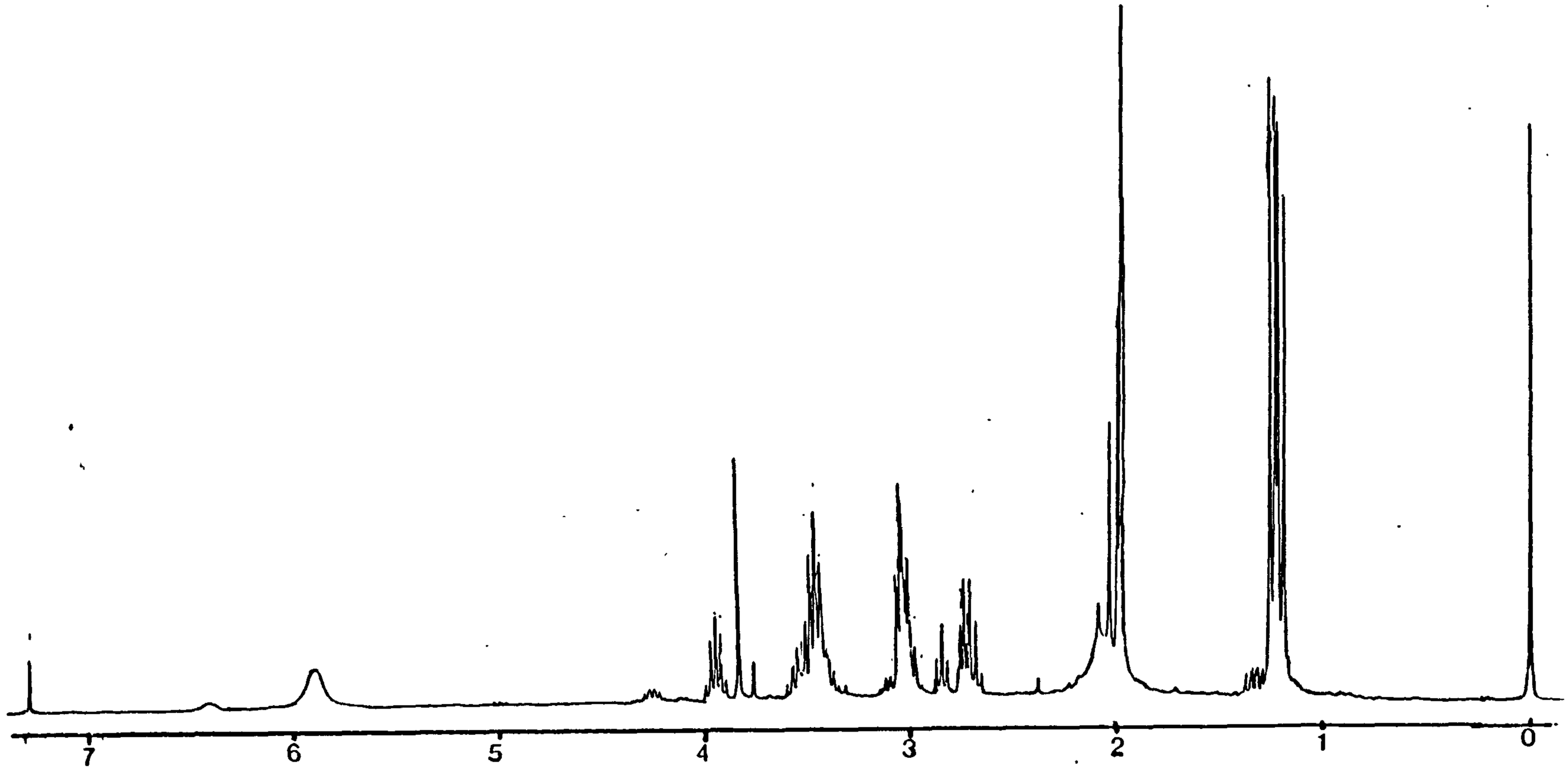


Figure 3.3 (cont) ^{1}H nmr spectrum of racemic NAC thioester of 3-hydroxy-2-methylbutanoic acid.

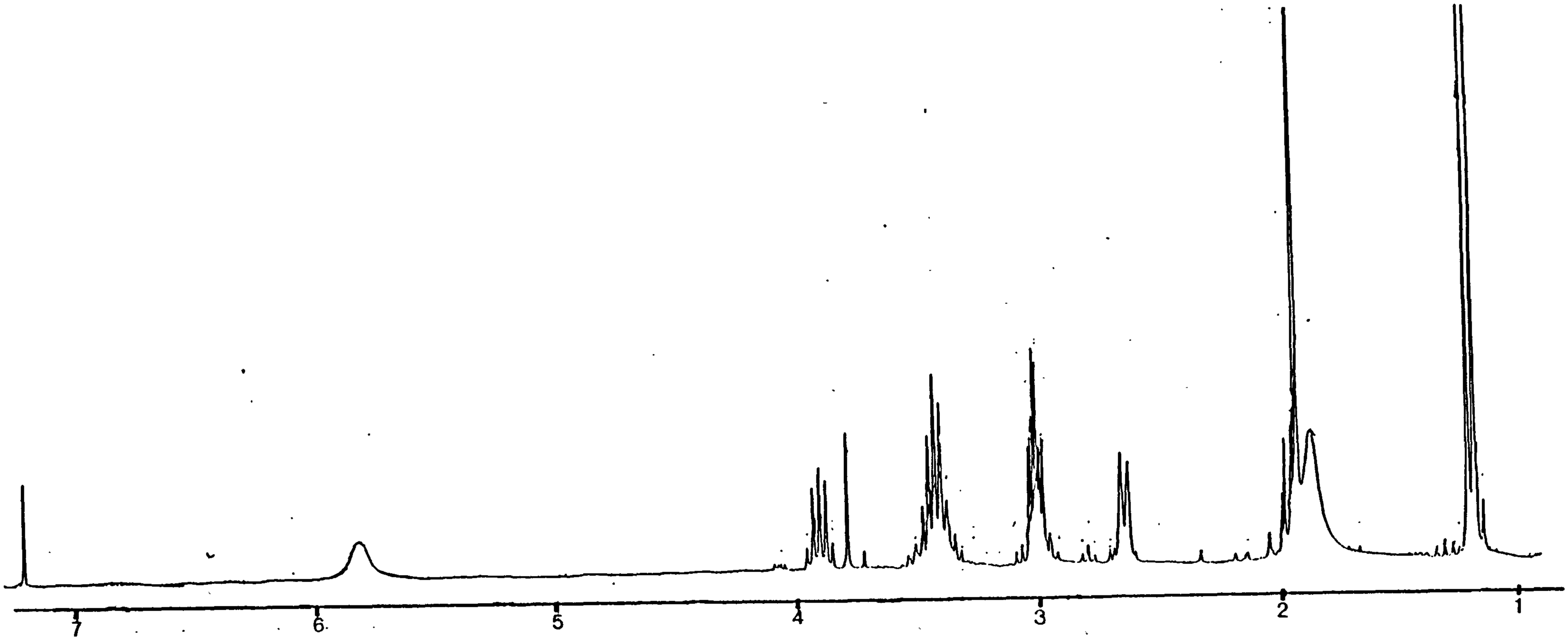
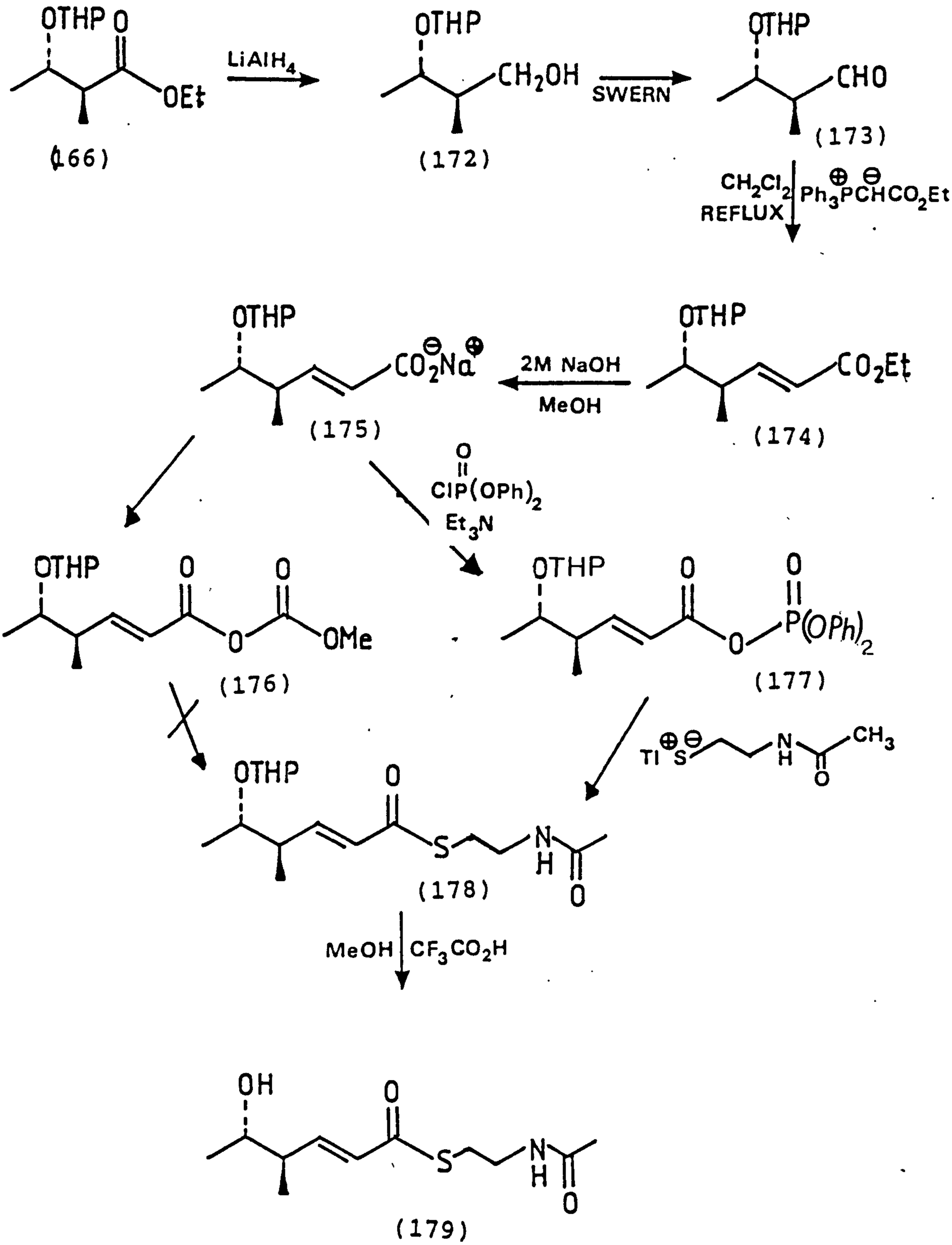


Figure 3.3 ^{1}H nmr spectrum of NAC thioester of (2S,3S)-[2'-$^{2}H_{3}$]-3-hydroxy-2-methylbutanoic acid.

literature value[49(b)] for this compound (163), $[\alpha]_D^{22}$ = +19.1°. For the product from methylation of the alcohol generated from the fresh yeast reduction, $[\alpha]_D^{20}$ = 20.4°, giving an optical purity of 94%. For the methylated product using the alcohol generated from the dried yeast reduction, $[\alpha]_D^{20}$ = 22.7°, giving an optical purity of 84%. For conversion to the NAC thioester, the hydroxyl group was protected as a THP ether and then an analogous sequence of steps to that outlined previously[46] of the racemic thioester (170) was followed, ie. activation as a mixed anhydride (168), coupling to N-acetylcysteamine and finally deprotection. Purification of the resulting NAC thioester (170) was again achieved by flash chromatography.

For incorporation studies, this chain assembly intermediate, (2S,3S)-3-hydroxy-2-methylbutanoic acid was synthesised as the ethyl ester, NAC ester and sodium salt, with a deuterium label in the C-2 methyl group. This label was introduced from CD_3I in the stereospecific methylation reaction. The ^1H nmr of this optically active thioester is shown in figure 3.3. and shows that in the deuterium labelled material, the methyl of the butane chain signal appears as a doublet centered at δ0.98, *cf.* in the unlabelled material two doublet signals are observed for the C-2 methyl group and the methyl group of the butane chain. The enantiomeric purity of this product was checked by ^1H nmr methods using (R)-(-)-TFEA as the CSA. In this experiment a 10:1 addition of CSA to solute was used. For this NAC thioester (170), non-equivalence is readily observed in the amide methyl signal. In the racemic material, the amide methyl appears



(166) (172) (173) (175) (174) (176) (177) (178) (179)

Scheme 3.6

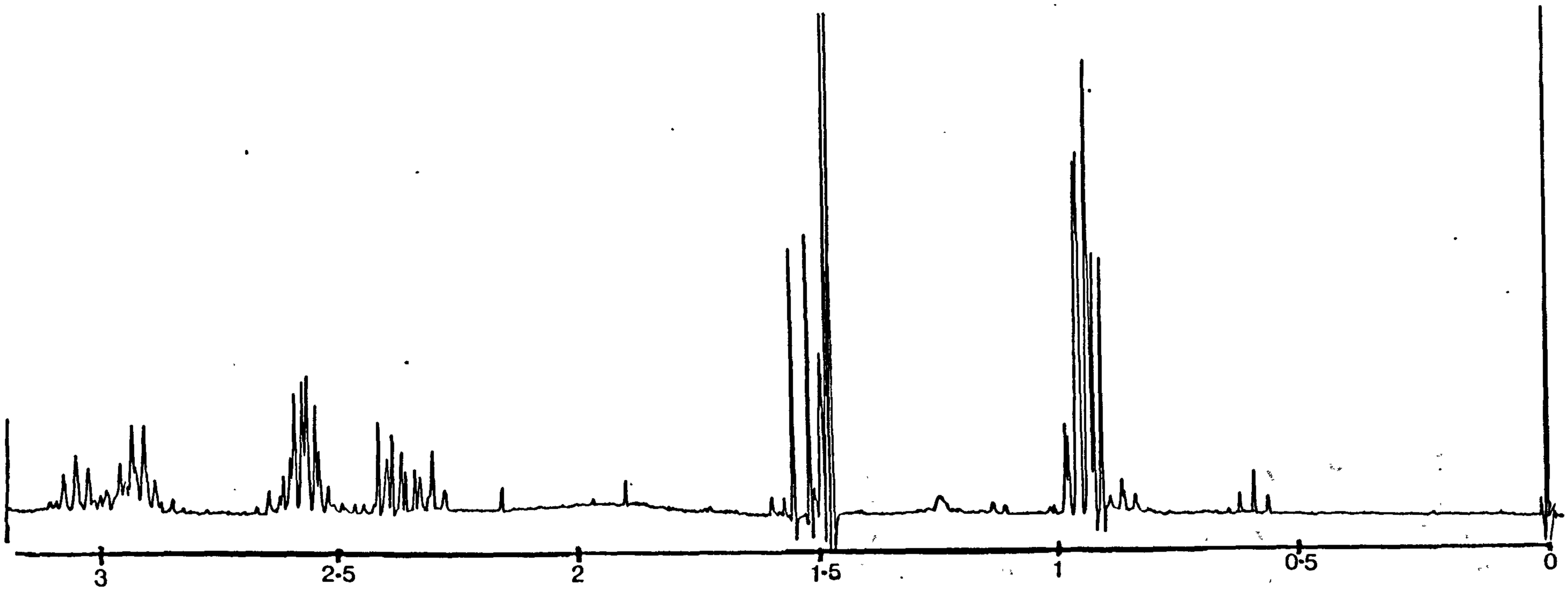


Figure 3.3 (cont) ^{1}H nmr spectrum of racemic NAC thioester of 3-hydroxy-2-methylbutanoic acid with the addition of CSA reagent (TFEA).

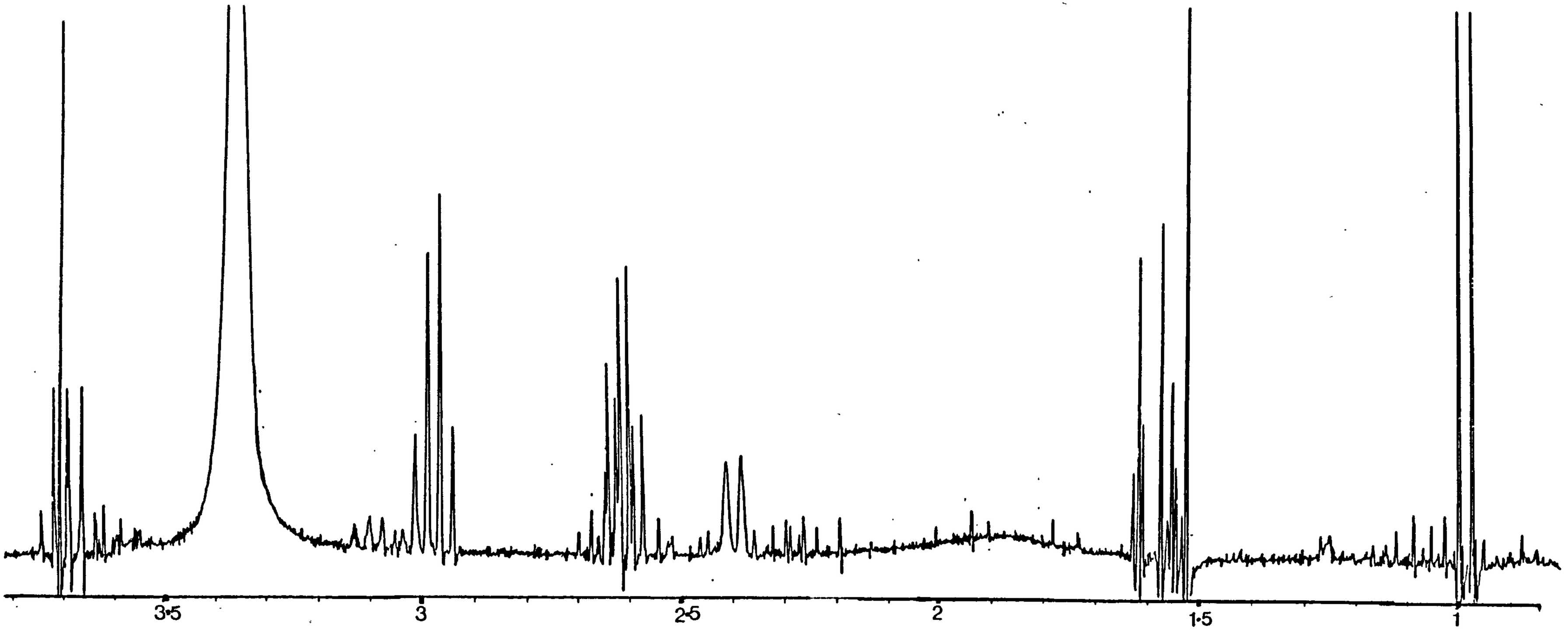


Figure 3.3 ^{1}H nmr spectrum of NAC thioester of (2S,3S)-[2'-$^{2}H_3$]-3-hydroxy-2-methylbutanoic acid with the addition of CSA reagent (TFEA).

as two singlet signals centered at δ1.49 and in the chiral material, this signal appears as one singlet at δ1.53. This indicates this material to be enantiomerically pure. Also, since there is only one doublet signal for the methyl of the butane chain further supports that no undesired epimerisation of the C-2 and C-3 chiral centres had occurred.

The synthesis of the NAC thioester of (2E,4S,5S)-5-hydroxy-4-methylhex-2-enoic acid (179) is shown in scheme 3.6. Again, racemic starting material, ethyl 3-hydroxybutyrate (162) was used intially, to test this route. The first two steps, were as described in the previous synthesis of the NAC thioester deriviative of 3-hydroxy-2-methylbutanoic acid (170). Various forms of protection of the hydroxyl group on C-3 were tried, to find one that could be readily cleaved using very mild reaction conditions, but which would remain intact throughout the synthesis. The groups tried were; trimethyl silyl ether, ^{t}butyl-dimethyl silyl ether, O-(1-ethyoxyethyl) and finally THP ether. The only protecting group, of these tried that remained in place throughout the synthesis was the THP ether. Both silyl ethers failed to survive the reaction conditions of the lithium aluminium hydride reduction, and the O-(ethoxyethyl) protection from ethyl vinyl ether did prove to be more resilient but was readily removed when the ester group was hydrolysed.

Treatment of the THP ether protected ethyl 3-hydroxy-2-methylbutyrate (166) with lithium aluminium

hydride produced the alcohol (172) in very good yield. This was then oxidised to the corresponding aldehyde (173). This oxidation reaction proved to be difficult as the alcohol (172) was resisent to both pyridinium dichromate (PDC) and pyridinium chlorochromate (PCC) oxidation conditions. Swern oxidation[11(e)], using oxalyl chloride and dimethyl sulphoxide (DMSO) eventually provided the desired aldehyde (173). This was then subjected to a Wittig reaction[56] with triphenyl carbethoxymethylidene phosphorane to generate the unsaturated ester (174). The yield from this reaction was intially poor, but changing the solvent from benzene to dichloromethane and increasing the reaction time to four days at reflux, did provide (174) in reasonable yield. Intially, the previous NAC thioester coupling reactions[46] were tried, *ie.* hydrolysis of the ester (174) to the free acid (175), and then activation as the mixed anhydride (176) - both reactions went in good yield - and then coupling to N-acetylcysteamine. The ^{1}H nmr spectrum of the reaction product, revealed that the vinyl signals at δ6.1-6.2 and δ6.9-7.0 were very weak. This indicated that the sulphur of the N-acetylcysteamine group, which is a 'soft' nucleophile, had attacked the carbon carbon double bond in a Michael type reaction. This is the 'soft' electrophilic site in this molecule and so this reaction should have been predicted. Some of the desired thioester (179) was isolated from the reaction products by preparative tlc. This problem of the Michael reaction was solved by changing the nucleophilicity of the sulphur. Instead of using N-acetylcysteamine, the Thallium (I) salt of N-acetylcysteamine was used[50,55,57], and the coupling agent was changed to chlorodiphenyl

phosphinite. The sulphur now carries a full negative charge, and so is a much 'harder' electrophile and so attacks the 'hard' nucleophilic carbonyl site in preference to the 'soft' electrophilic carbon to carbon double bond. After deprotection using trifluoroacetic acid in methanol, and purification by flash chromatography pure NAC thioester (179) was formed. Nmr analysis was obtained for each intermediate compound, with the spectra being complex due to the presence of the THP ether protecting group. Figure 3.4 shows the 300MHz nmr spectrum of this thioester (179). It clearly shows the thioester groups at δ3.1 and δ3.4, and vinyl resonances at δ6.1-6.2 and δ6.9-7.0, with J=15Hz for a trans double bond. The resonance for H-2 at δ6.05 shows a very small splitting (1Hz) due to long range coupling to H-3 which is four bonds away. This is not uncommon, especially since one of the intervening bonds is a π bond. Mass spectrometry shows a molecular ion at 246 as expected.

This NAC thioester (179), could now be synthesised in optically active form using this route, with ethyl (3S) 3-hydroxybutyrate as the starting material. For incorporation studies, this NAC thioester could be synthesised in labelled form, with methylation using deuterated methyl iodide or with a ^{13}C label in the triphenyl carbethoxymethylidene phosphorane as being possible ways of introducing the required isotopic label, and the diastereomeric purity of this intermediate, could be found using the CSA method outlined previously.

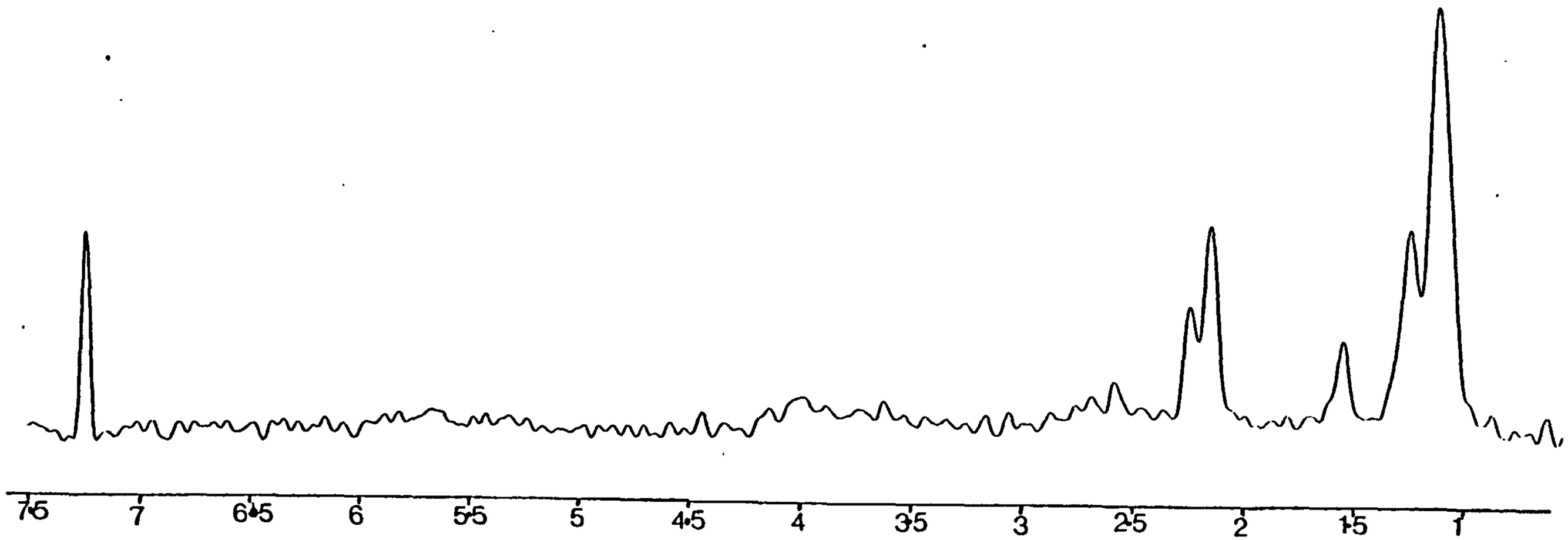


Figure 3.6 ^{2}H nmr spectrum of methyl pseudomonate labelled from sodium [2-$^{2}H_3$]acetate.

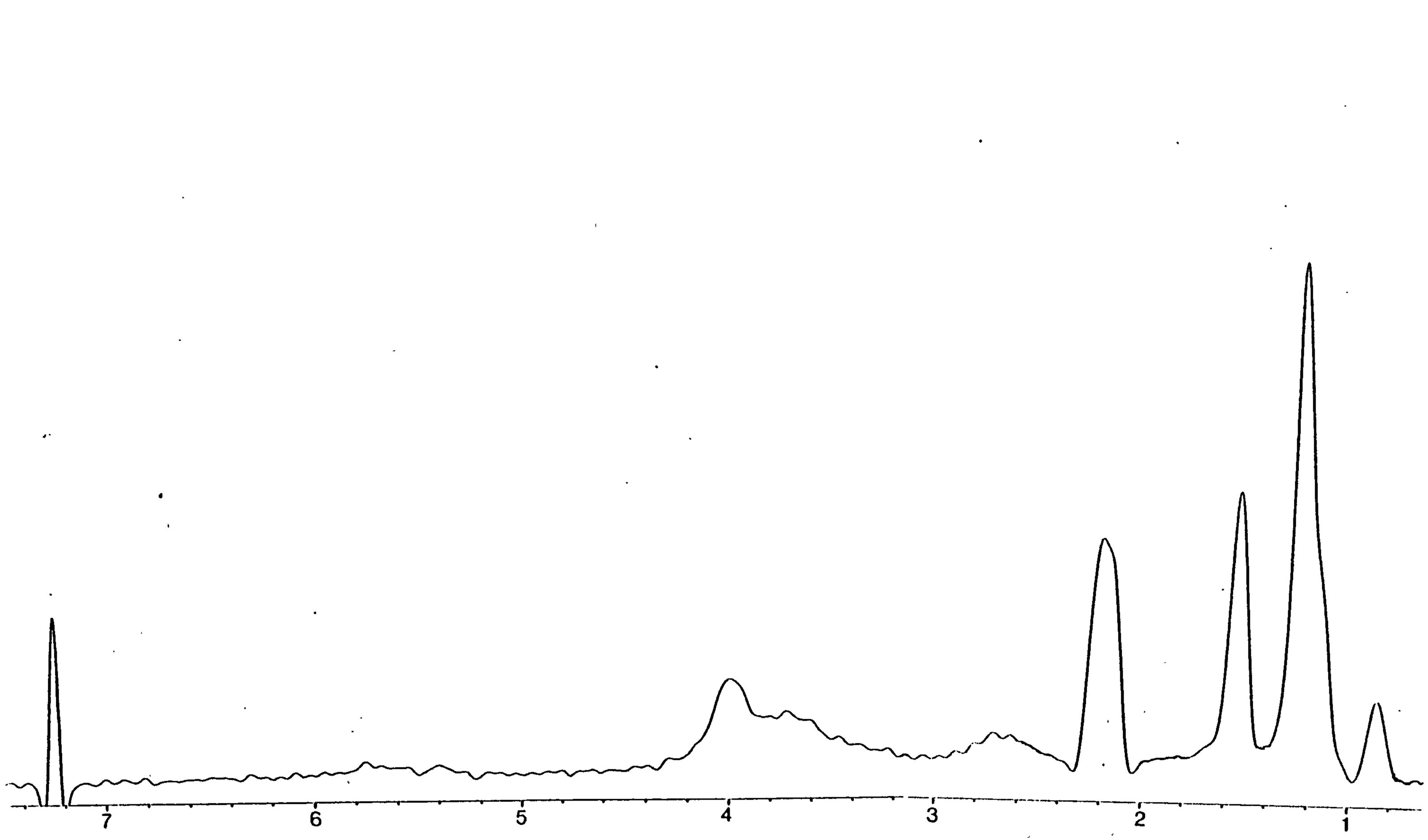


Figure 3.5 ^{2}H nmr spectrum of generally deuterated methyl pseudomonate.

TABLE 3.1

55.28 MHz ^{2}H nmr spectra of methyl pseudomonate isolated from incorporation studies using ^{2}H-labelled precursors.

PRECURSOR	δ (ppm)	ASSIGNMENT
Sodium acetate	1.11	14-Methyl
	1.24, 1.54	9-Hydroxynonanoic acid side chain resonances
	2.14	15-Methyl
$^{2}H_2O$	0.86	17-Methyl
	1.22, 1.53	14-Methyl and 9-hydroxy-nonanoic acid side chain resonances
	2.19	15-Methyl
	4.28	Monic acid resonances
Compound (146)		
Sodium salt	1.09	Breakdown product
Ethyl ester	1.11, 1.31, 1.79	Breakdown product
NAC thioester	1.12, 1.34, 3.21	Breakdown product

3.4 INCORPORATION STUDIES ON CHAIN-ELONGATION ASSEMBLY INTERMEDIATES, IN *P. fluorescens*

The proposed involvement of (2S,3S)-3-hydroxybutanoic acid (170) as a chain assembly intermediate in the biosynthetic pathway to pseudomonic acid has been tested. This proposed intermediate was fed in three forms ie. as the NAC thioester, as the ethyl ester and as the sodium salt. Deuterium at the C-2 methyl was the isotopic label used and ^{2}H nmr spectroscopy was used to determine whether this precuror was incorporated intact.

For comparison, an intial experiment to obtain a generally deuterated sample of methyl pseudomonate was carried out. This was achieved by growing the culture in a second stage medium containing 5% $^{2}H_2O$. The ^{2}H nmr spectrum of the isolated labelled methyl pseudomonate is shown in figure 3.5, and assignment (table 3.1) was made by comparison with the ^{1}H nmr spectrum of the metabolite. Sodium [2-$^{2}H_3$]acetate was also fed to the culture to provide the ^{2}H nmr spectrum shown in figure 3.6, and the assignment is detailed in table 3.1. This was used to assess the level of intact incorporation of the chain assembly intermediates labelled with deuterium, since breakdown to acetyl CoA then subsequent re-incorporation is the most likely route for label to enter the metabolite from catabolism of the precursor.

The results of feeding (2S,3S)[2'-$^{2}H_3$]-3-hydroxy-2-methylbutanoic acid in the three forms, specified above, to *P. fluorescens* and analysis by

^{2}H nmr spectroscopy (table 3.1) revealed that none of these compounds were incorporated intact. Catabolism of precursors larger than acetate or propionate has been an inherent problem with whole cell studies of polyketide metabolism[59]. It was thought, that precursors as their NAC thioester deriviatives could more easily undergo transesterification with the thiol group on an enzyme than an oxy ester[60], but this compound (170) does not seem to have survived the fermentation conditions.

3.5 CONCLUSIONS AND FURTHER WORK

These synthetic approaches to the NAC thioesters of the proposed chain elongation intermediates to pseudomonic acid are suitable for the convenient introduction of isotopic labels, at the positions indicated in the text. These NAC thioesters could also be used to test other biosynthetic pathways. The incorporation of the NAC thioesters of [2,3-$^{13}C_2$]acetoacetate and (2E,4S,5S)[4'-$^{2}H_3$]-5-hydroxy-4-methylhex-2-enoic acid remain to be studied, along with the corresponding oxy esters and sodium salts of these compounds. The diastereomeric purity of the latter compound (179), formed by the route detailed in scheme (3.6) remains to be checked. The intial results from incorporation of the NAC thioester of (2S,3S) [2'-$^{2}H_3$]-3-hydroxy-2-methylbutanoic acid and the corresponding ethyl ester and sodium salt compounds have indicated that this whole cell system might not be suitable for testing this stepwise chain assembly to the final polyketide metabolite since none of these compounds were

incorporated intact. Further work to optimise the feeding conditions might increase the chances of achieving intact incorporation.

EXPERIMENTAL

Chapter three

Experimental

General experimental details are as detailed on page 45.

SYNTHESIS OF CYCLOHEXANE THIOESTER OF ACETOACETATE

Meldrum's acid, (2,2-dimethyl-1,3-dioxane-4,6-dione), (159) (0.5g, 3.5mmol) in 25ml of dry dichloromethane was reacted with acetyl chloride (0.33g, 0.3ml, 4.2mmol) and pyridine (0.54g, 0.55ml, 6.8mmol), under a N_2 atmosphere. This solution was kept at 0^oC for 1h and then allowed to warm to room temperature and was stirred for a further 1h. After this time, the solution was washed with dilute HCl, water and dried over anhydrous $MgSO_4$. The solvent was evaporated *in vacuo* to yield the acyl deriviative of Meldrum's acid (160) in a yield of 0.28g (1.5mmol, 43%).

δ_H ($CDCl_3$, 60MHz) 1.8 (6H, s, 2x$C\underline{H}_3$), 2.1 and 2.25 (3H, 2xs, tautomeric signals for $C\underline{H}_3$-C=C-CO), 2.7 (1H, s, O$\underline{H}$).

This acylated Meldrum's acid (160), (0.28g, 1.5mmol), was reacted with cyclohexane thiol (0.42g, 0.44ml, 3.6mmol, 2.4 equiv.), under a N_2 atmosphere, in refluxing dry benzene for 3h. The resulting solution was cooled and the benzene evaporated *in vacuo*. The product was purified by preparative tlc using 3% ether in petrol ether ($30-40^o$) as the running solvent. This yielded the cyclohexane thioester of acetoacetate (161) as an orange oil (0.12g, 0.60mmol, 40%).

δ_H ($CDCl_3$, 60MHz) 1.2-2.2 (10H, br, $C\underline{H}_2$ ring protons), 2.3

(3H, s, $C\underline{H}_3CO$), 2.6-3 (br, $O\underline{H}$), 3.7 (2H, s, $C\underline{H}_2CO$), 5.4 (olefinic proton).

SYNTHESIS OF THE N-ACETYLCYSTEAMINE THIOESTER OF ACETOACETATE

^{n}Butyl lithium (13.9ml of a 1.6M solution in hexanes, 2.1 equivalents) was added to a stirred solution of HMDS (5ml, 23.5mmol, 2.1 equivalents) in dry THF (5ml) at -78°C, under a nitrogen atmosphere. After being stirred for 5min at -78°C, the solvents were removed *in vacuo* at room temperature. The resulting white solid, was re-dissolved in dry THF (30ml) and cooled to -78°C, under a nitrogen atmosphere. Ethyl acetate (1g, 12.7mmol) was added slowly to the reaction mixture and this was then stirred for a further 20min. Acetyl chloride (1.1ml), as a solution in 1ml of dry THF, was added slowly, to the generated enolate and the mixture stirred for 15min at -78°C. 2M HCl (15ml) was added and the aqueous and organic layers were separated, and the aqueous layer further extracted with diethyl ether (3x 20ml). Organic extracts were combined, dried over anhydrous $MgSO_4$ and evaporated to dryness. This yielded ethyl acetoacetate (151), 1.55g (11.9mmol, 94%) δ_H ($CDCl_3$, 250.133 MHz) 1.3 (3H, t J=4.8Hz, $C\underline{H}_3$), 1.9 ($C\underline{H}_3$ of enol form), 2.3 (3H, s, $C\underline{H}_3CO$), 3.45 (2H, s, $C\underline{H}_2CO$), 4.2 (2H, q J=7.2Hz, $OC\underline{H}_2$), 5.0 (olefinic $C\underline{H}$).

Ethyl acetoacetate (151), (10g, 9.7ml, 77mmol) was reacted with (9.9g, 154mmol, 2 equivalents) of ethylene glycol in

the presence of a catalytic amount of toluene-4-sulphonic acid (PTSA) in dry benzene (70ml) for 24h at reflux using a Dean-Stark apparatus. After cooling the solution, it was washed successively with; 5% $NaHCO_3$ solution (50ml), water (2x 50ml) and then dried over anhydrous $MgSO_4$. The solvent was evaporated *in vacuo* to yield ethyl acetoacetate ethylene ketal (152) as a yellow oil, (10.1g; 58mmol; 75%).

δ_H ($CDCl_3$, 60MHz) 1.2 (3H, t J=7Hz, $C\underline{H}_3$), 1.45 (3H, s, $C\underline{H}_3$), 2.65 (2H, s, $C\underline{H}_2CO$), overlapping signals at 3.95 (4H, s, $2xC\underline{H}_2O$) and 4.15 (2H, q J=7Hz, OCH_2)

The protected ester (152), (1g, 5.7mmol) was stirred at room temperature in a solution of KOH (1.44g, 4.5 equivalents) in water/ethanol (15ml of a 1/1, v/v mixture). The solvent was evaporated and water was added and this aqueous solution was then extracted into hexane (3x 20ml). The hexane extracts were combined and back extracted with water. All the aqueous extracts were combined and the pH adjusted to 8 with 1M HCl before the water was removed as an ethanol azeotrope to yield a white solid.

The resultant sodium acetoacetate ethylene ketal (153) was used directly in the next reaction. This white solid was suspended in dry THF (20ml) and methyl chloroformate (0.86ml, 12.5mmol, 2.2 equivalents) and one drop of triethylamine were added. The resulting suspension was stirred vigorously at room temperature for 24h. Celite was added and the mixture was filtered and the filtrate concentrated *in vacuo* to yield the mixed anhydride of methyl formate acetoacetate ethylene ketal (154), (0.97g, 4.75mmol,

83%).

δ_H ($CDCl_3$, 60MHz) 1.5 (3H, s $C\underline{H}_3$), 2.8 (2H, s, $C\underline{H}_2CO$), 3.9 (3H, s, $OC\underline{H}_3$), 4.0 (4H, s, $2xC\underline{H}_2O$).

ETHANETHIOIC ACID (S)-[2-(ACETYLAMINO)ETHYL]ESTER (N,S-DIACETYLCYSTEAMINE) (156), AND N-(2-MERCAPTOETHYL)ACETAMIDE (N-ACETYLCYSTEAMINE (157)

To a three-necked 250ml round bottomed flask equipped with a pH electrode and two addition funnels was added 5.68g (50mmol) of 2-mercaptoethylamine hydrochloride in 40ml of water. One addition funnel was filled with 14.14ml (150mmol) of acetic anhydride and the other with ca. 40ml of 8M KOH. After the contents of the flask had been cooled by means of an ice bath and the pH had been adjusted to 8.0 (by addition of some of the KOH solution), acetic anhydride was added dropwise along with sufficient KOH solution to maintain the pH at approximately 8.0. After all the acetic anhydride had been added, the pH of the reaction mixture was adjusted to 7.0 by addition of 2M HCl solution, and the mixture was stirred for 1h. NaCl was then added until the solution was saturated and this mixture was extracted with dichloromethane (5x 50ml). The combined extracts were dried and concentrated to yield 8.0g (98%) of a colourless oil, (156).

δ_H ($CDCl_3$, 60MHz) 2.0 (3H, s, $C\underline{H}_3CONH$), 2.4 (3H, s, $C\underline{H}_3COS$), 2.9-3.2 (2H,m, $C\underline{H}_2S$), 3.3-3.6 (2H,m, $C\underline{H}_2NH$), 6.8 (1H, br s, $N\underline{H}$).

Compound (157), was generated as follows; To a 250ml round

bottomed flask containing 3.06g (19mmol) of N,S-diacetylcysteamine in water (100ml), cooled to 10°C, was added solid KOH (3.55g, 63.3mmol). This solution was stirred at room temperature for 0.5h, under a nitrogen atmosphere. After adjusting the pH to 7 (with 1M HCl) and saturating the solution with NaCl, it was extracted with dichloromethane (5x 50ml). The combined organic extracts were dried, and evaporated to yield a colourless oil, (157) which was used immediately in the following reaction.

δ_H ($CDCl_3$, 60MHz) 1.45 (1H, t J=7Hz, SH), 2.0 (3H, s, CH_3CO), 2.45-2.9 (2H, m, CH_2SH), 3.2-3.6 (2H, m, CH_2NH), 7.0 (1H, br s, NH).

The mixed anhydride (154), (0.97g, 4.75mmol) was reacted with N-acetylcysteamine (157), (1.31g, 11.1mmol, 2.4equivalents) in dry THF (20ml) and triethylamine (1.4ml, 9.5mmol, 2 equivalents). This solution was stirred at room temperature for 24h. Ethyl acetate was added, and the solution washed with cold 1M KOH. The aqueous extract was back extracted with some more ethyl acetate and the organic extracts were combined, dried and evaporated *in vacuo* to yield the N-acetylcysteamine thioester of acetoacetate ethylene ketal (155) as a thick yellow oil. (1.03g, 4.2mmol, 88%).

δ_H ($CDCl_3$, 60MHz) 1.4 (3H, s, CH_3), 2.0 (3H, s, CH_3CO), 2.95 (2H, s, CH_2CO) and this signal overlaps with 3.0-3.3 (2H, m, CH_2S), 3.4-3.7 (2H, m, CH_2N), 4.0 (4H, s, 2xCH_2O), 6.2 (1H, br S, NH).

The protected thioester (155), (0.5g, 2mmol) was reacted with toluene-4-sulphonic acid (0.42g, 2.2 mmol, 1.1 equivalents) in dry acetone (10ml) at reflux for 2.5h. This yielded NAC thioester of acetoacetate (144), 0.247g (1.2mmol, 60%) as an oil which was purified by flash silica column chromatography using 100ml of hexane/ethyl acetate (1/4) and 1 drop of triethylamine and then 100ml of 100% ethyl acetate and 1 drop of triethylamine. This provided pure thioester (144) as a yellow oil (0.28g, 1.4mmol, 70%).

δ_H ($CDCl_3$, 80.13MHz) 1.8 (three overlapping s, $C\underline{H}_3$, enol form of the b-ketone and amide), 2.1 (6H, s, $2xC\underline{H}_3CO$), 2.9 (2H, m, $C\underline{H}_2S$), 3.3 (2H, m, $C\underline{H}_2NH$), 3.6 (2H, s, $C\underline{H}_2CO$), 5.3 (olefinic C$\underline{H}$), 6.2 (br s, N$\underline{H}$).

Mass spectrum m/z 204 (M^+)
Calcd. for $C_8H_{14}NO_3S$ (MH^+) 204·06943; found 204·06940.

SYNTHESIS OF THE N-ACETYLCYSTEAMINE THIOESTER OF (2S,3S)-3-HYDROXY-2-METHYLBUTANOIC ACID

Fresh baker's yeast (100g) was dispersed into a solution of sucrose (1000g) in 8000ml of tap water contained in a fermentor. After 0.5h the yeast was activated and ethyl acetoacetate (22.1g, 21.5ml, 0.17mol) was added in portions over a 8h period. This suspension was then allowed to stir, with aeration at a temperature of 28°C for 4 days. The cells were removed by centrifugation and the aqueous phase extracted with diethyl ether (81), dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. After distillation under reduced pressure (45°C, 0.7mbar), ethyl (3S)-3-hydroxybutyrate (162) as a colourless oil was

obtained (8.23g, 0.063mol, 37%).

δ_H (d_6 benzene, 200.130MHz) 0.9 (3H, t J=5Hz, C$\underline{H}_3$), 1.0 (3H, d J=5Hz, C$\underline{H}_3$), 2.1-2.18 (2H, m, AB of an ABX, C$\underline{H}_2$), 3.9 (1H, br s, O$\underline{H}$), 3.8 (2H, q J=5Hz, OC$\underline{H}_2$), 4-4.1 (1H, m, X of an ABX, H-3).

Calculation of specific rotation:

Weight of compound used = 0.157g in 5ml $CHCl_3$
Readings = 1.146, 1.146, 1.145
Average = 1.146
Temperature = 20°C

$$\text{Specific rotation} = \frac{\text{observed rotation (degrees)}}{\text{length (dm)} \times \text{g/cm}^3}$$

$$= \frac{1.146}{1 \times 0.0314}$$

$$= \underline{+36.5}^{\circ}$$

Lit[48(a)] $[\alpha]_D^{20}$ = $\underline{+38.5}^{\circ}$ ($CHCl_3$, c 1).

The yeast reduction was repeated using 5x 500ml shake flasks and active dried yeast (single strain, *Saccharomyces cerevisiae*). Each flask contained yeast (10g) and glucose (10g) in 100ml of distilled water. Fermentation and feeding conditions (*ie.* 1g, 1.1ml ethyl acetoacetate per flask) were as for the fresh yeast. The cells were removed by filtration through celite. The resulting aqueous extract was extracted into diethyl ether (5x 100ml), dried over anhydrous $MgSO_4$ and the solvent evaporated *in vacuo*. After distillation under reduced pressure, as above, **ethyl-(3S)-3-hydroxybutyrate (162)**, as a colourless oil was obtained, (3.26g, 24.7mmol, 64%).

δ_H ($CDCl_3$, 300.133MHz) 1.24 (3H, t J=6Hz, C$\underline{H}_3$), 1.34 (3H, t

J=6Hz, $C\underline{H}_3$), 2.45 (2H, m, AB of ABX, $C\underline{H}_2$), 4.1-4.25 (2H, q J=6Hz, $OC\underline{H}_2$), overlaps with (1H, m, X of ABX, H-3).

Calculation of specific rotation:
Weight of compound used = 0.134g in 10ml $CHCl_3$
Readings = 0.484, 0.483, 0.484
Average = 0.484
Temperature = 20°C

$[\alpha]_D^{20} = \underline{+36.1}^{\circ}$

To a stirred solution of di-isopropylamine (10.9ml, 77mmol) in dry THF, under a N_2 atmosphere at -78°C was added ^{n}butyl lithium (46ml 1.4M solution in hexanes). The resulting solution was allowed to warm to 0°C and then re-cooled to -50°C and ethyl (S)-3-hydroxybutyrate (4ml, 29.8mmol) was added quickly. After the addition was complete, the solution was allowed to warm slowly to -20°C and then was re-cooled to -78°C. Methyl iodide (8ml, 128mmol) in dry THF (5ml) was added and the resulting solution allowed to warm to room temperature and was stirred at room temperature overnight. Saturated NH_4Cl solution (10ml) was added and the THF evaporated. The residue was extracted into diethyl ether (5x 50ml), and these organic extracts were combined washed with brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The resulting brown oil was purified by Kugelrohr distillation (40°C, 0.07mbar). This yielded ethyl (2S,3S)-3-hydroxy-2-methylbutyrate (163), as a colourless oil (2.53g, 66%).

δ_H ($CDCl_3$, 300.133MHz) 0.9 (3H, t J=6Hz, $C\underline{H}_3$), 1.0 (3H, d J=6Hz, $C\underline{H}_3$), 1.05 (3H, d J=6Hz, $C\underline{H}_3$),

2.3 (1H, dq J=6Hz, H-2), 3.75 (1H, dq J=6Hz, H-3), 3.9 (2H, q J=6Hz, $OC\underline{H}_2$).

Calculation of specific rotation:

Weight of compound used = 0.300g in 10ml $CHCl_3$
Readings = 0.613, 0.622, 0.611
Temperature = 20°C

$[\alpha]_D^{20} = \underline{+20.4}^{\circ}$

Lit.[49(b)] $[\alpha]_D = \underline{+19.1}^{\circ}$ ($CHCl_3$, c 1.3)

The ethyl (2S,3S)-3-hydroxy-2-methylbutyrate generated from using the dried baker's yeast gave the following optical rotation:

Weight of compound used = 0.0608g in 10ml $CHCl_3$
Readings = 0.138, 0.138, 0.139
Temperature = 20°C

$[\alpha]_D^{20} = \underline{+22.7}^{\circ}$

To ethyl (2S,3S)-3-hydroxy-2-methylbutanoate (163), (1.51g, 10.3mmol) in dichloromethane at 0°C, was added dihydropyran (4.6ml, 4.4g, 52.5mmol, 5equiv.) followed by trifluoroacetic acid (1 drop). This solution was stirred at room temperature overnight. Volatile materials were evaporated to yield the tetrahydropyran ether (165) in quantitative yield, (2.46g). this was used directly without purification. To a solution of (165), (2.43g, 10.4mmol) in dry methanol (20ml), cooled to 0°C was added 2M NaOH (7.6ml, 15.15mmol, 1.5equiv). This was stirred at room temperature overnight. The methanol was evaporated and distilled water (20ml) was added to the residue which was extracted with hexane (3x20ml). The hexane extracts were combined and back

extracted with water. All the aqueous extracts were combined and the pH adjusted to 8 with 1M HCl. The water was removed as an ethanol azeoptrope to yield the sodium salt of the THP ether of 3-hydroxy-2-methylbutyrate (167) as white solid. This was used directly in the next stage of the reaction. This white solid was suspended in dry THF (35ml) and methyl chloroformate (2.3ml, 16.3mmol, 2.2 equiv.) and triethylamine (1 drop) was added. This suspension was stirred vigorously at room temperature for 24h. Celite was added and the mixture filtered. The filtrate was concentrated *in vacuo* to yield the mixed anhydride (168), (2.36g, 9.1mmol, 88%).

δ_H ($CDCl_3$, 80MHz) *Inter alia*, (two 1H, m, acetal protons), 3.8 (3H, br s, $OC\underline{H}_3$).

To the crude mixed anhydride (168), (2.355g, 9mmol) as a solution in dry THF, cooled to 0^oC, was added N-acetylcysteamine (2.3g, 27mmol, 3 equiv.), generated as described previously, and triethylamine (7.6ml, 18mmol, 2 equiv.). This mixture was stirred at room temperature for 20h. It was then diluted with ethyl acetate (100ml) and washed with cold 1M KOH (2x40ml). The aqueous extract was back extracted with ethyl acetate. Organic extracts were combined, dried and evaporated to yield the protected NAC thioester of 3-hydroxy-2-methylbutanoic acid (169), (2.41g, 7.8mmol, 86%). Deprotection of (169) to yield NAC thioester (2S,3S)-3hydroxy-2-methylbutanoic acid (148) was achieved by reacting (147) (2.4g, 7.84mmol) with a catalytic amount of trifluoroacetic acid (0.04ml) in methanol (20ml). The solution was stirred at room temperature for 16h and then

the volatile materials were evaporated *in vacuo* to yield (170) as a yellow oil (1.66g, 7.6mmol, 97%). This crude NAC thioester was purified by flash silica column chromatrography. The eluting solvents were; ethyl acetate: hexane (4/1), and 1 drop triethylamine (50ml) and then 100% ethyl acetate and 1 drop of triethylamine (200ml). This yielded pure NAC thioester (170) (1.29g, 5.9mmol, 76%).

δ_H ($CDCl_3$, 250.133MHz)	1.2 (3H, d J=7.1 Hz, $C\underline{H}_3$), 1.25 (3H, d J=6.3Hz, $C\underline{H}_3$), 1.98 (3H, s, $C\underline{H}_3CO$), 2.65-2.75 (1H, m, H-2), 3.0-3.1 (2H, m, $C\underline{H}_2S$), 3.4-3.5 (2H, dq J=7.1Hz, $C\underline{H}_2N$, 3.9 (1H, dq J=6.5Hz, H-3), 5.9 and 6.4 (2xbr s, O$\underline{H}$, and N$\underline{H}$).
Mass spectrum	m/z 220 (M^+), 45 (CO_2^+), 60 ($CH_3CONH_2^+$), 119 ($CH_3CON(CH_2)_2S^+$).

Calcd. for $C_9H_{18}NO_3S$ (mH^+) 220.10073 ; found 220.100700

SYNTHESIS OF THE NAC THIOESTER OF (2S,3S)-[2'-$_2H_3$]-3HYDROXY-2-METHYLBUTANOIC ACID

This compound was prepared as described, for the synthesis of (170), starting from ethyl (2S)-3-hydroxybutyrate (162), (3.5ml, 26.1mmol) formed by yeast reduction of ethyl acetoacetate, and alkylating (162) with CD_3I, (8ml, 128mmol, 99.5+ atom % D) to give ethyl (2S,3S)-[2'-$^2H_3$]-3-hydroxy-2-methylbutyrate (163), (2.531g, 66%). The ^1H nmr spectrum showed the signal at δ2.65 for H-2 as a broad doublet, and the absence of a doublet for the C-2 methyl group, as it is labelled with deuterium. The rest of this spectrum was as in the unlabelled material. Ethyl (2S,3S)-[2'-$^2H_3$]-3hydroxy-2methylbutyrate (163), (1.51g,

10.34mmol), was then converted to the corresponding NAC thioester (170), as outlined previously, in a yield of 1.29g (5.9mmol, 76%). All the ^1H nmr spectra of the intermediate compounds with the THP ether group were complex and complete spectral data is given for the final NAC thioester (170).

δ_H ($CDCl_3$, 250.133MHz) 1.25 (3H, d J=6.3Hz, $C\underline{H}_3$), 1.98 (3H, s, $C\underline{H}_3CO$), 2.3 (1H, br s, O$\underline{H}$), 2.65 (1H, br d J=7.5Hz, H-2), 3.0-3.1 (2H, m, $C\underline{H}_2S$), 3.4-3.6 (2H, m, $C\underline{H}_2N$), 3.9-4.0 (1H, dq J=6.5 Hz, H-3), 5.9 (1H, br, N$\underline{H}$).

SODIUM (2S,3S)-[2'-$^2H_3$]-3-HYDROXY-2-METHYLBUTANOIC ACID

Hydrolysis of labelled (163), (0.5g, 3.4mmol) to (164) was achieved using an automatic titrating apparatus. The starting pH was 12.0 and was maintained during the hydrolysis of the ester group by automatic addition of 1.0M NaOH solution, over a 24h period. This aqueous solution was then washed with hexane (3x 20ml). Hexane extracts were combined and back extracted with water. All aqueous extracts were combined and freeze-dried to yield (164), (0.42g, 3.39mmol).

δ_H ($CDCl_3$, 250.133MHz) 1.1 (3H, d J=6.4Hz, $C\underline{H}_3$), 2.25 (1H, d J=7Hz, H-2), 3.95 (1H, dq J=6.5Hz, H-3).

SYNTHESIS OF NAC THIOESTER OF RACEMIC 5-HYDROXY-4-METHYLHEX-2-ENOIC ACID.

The THP ether of ethyl 3-hydroxy-2-methylbutyrate (166),

(1g, 4.3mmol), in anhydrous diethyl ether (5ml) was added dropwise to a stirred suspension of $LiAlH_4$ (0.1g, mmol) in dry diethyl ether (15ml), under N_2 atmosphere. The reaction was stirred at room temperature for 1h and then refluxed for 2h. After cooling, water (0.5ml), 5% NaOH (0.5ml) and water (1ml) were added in succession and the resulting suspension was stirred for a few minutes, and then it was filtered through an anhydrous MgSO4 plug. The filtrate was concentrated *in vacuo* to yield the THP ether of 3-hydroxy-2-methylbutan-1-ol (172), (0.795g, 4.2mmol, 98%). δ_H ($CDCl_3$, 300.133MHz) *Inter alia* 0.95 (3H, 2xd J=6Hz, $C\underline{H}_3$), 1.2 and 1.3 (3H, 2xd J=6Hz, $C\underline{H}_3$), 2.8 (br s, O<u>H</u>).

To a solution of dry dichloromethane (25ml) and oxalyl chloride (1.0ml, 11mmol), cooled to -78°C was added dimethyl sulphoxide (1.70ml, 22mmol) in dry dichloromethane (5ml). The resulting solution was stirred for 2min and the alcohol (172), (1.57g, 8.4mmol) in dry dichloromethane (20ml) was added within 5min. The resulting solution was stirred for a further 15min at -78°C. Triethylamine (7.0ml, 50mmol) was then added and the mixture then stirred for a further 5min at -78°C, before being allowed to warm slowly to room temperature. Water (50ml) was added and the layers separated. The aqueous layer was re-extracted with dichloromethane (50ml). Organic extracts were combined and washed with brine, dried and evaporated to a small volume. This residue was taken up in some more dichloromethane and washed successively with 1% HCl, water, dilute (5%) $NaHCO_3$

solution, water and then dried and evaporated to dryness. This yielded the THP ether of 2-hydroxy-3-methylbutan-1-al (173), (0.98g, 5.3mmol, 63%).

δ_H ($CDCl_3$, 300.133MHz) *Inter alia* 1.05 and 1.1 (3H, 2xd J=6Hz, C$\underline{H}_3$), 1.19 and 1.3 (3H, 2xd J=6Hz, C$\underline{H}_3$), 2.45-2.6 (1H, m, H-2), 4.0-4.1 (1H, m, H-3), 9.73, 9.77 (1H, 2xd J=3Hz, C$\underline{H}$O) and minor diastereoisomers at 9.78 and 9.81.

Ethyl bromoacetate (13.3ml, 20g, 0.12mol) and triphenylphosphine (31.44g, 0.12mol) in toluene (100ml) were mixed and stirred at room temperature overnight. The resulting white precipitate was filtered and dried to yield ethoxycarbonylmethylenetriphenyl phosphonium bromide, 50.6g (0.12mol, 97%).

Mp 156-159°C (lit. value[56] = 158°C)

δ_H ($CDCl_3$, 60MHz) 0.33 (3H, t, J=8Hz, C$\underline{H}_3$), 3.31 (2H, q, J=8Hz, C$\underline{H}_2$), 4.78 (1H, bd, J=14Hz, C$\underline{H}$), 6.75-7.5 (15H, m, aromatic protons).

This salt, ethoxycarbonylmethylenetriphenyl phosphonium bromide, (1g, 2.51mmol) was dissolved in water (50ml) and titrated against 0.05M NaOH solution with vigorous stirring using phenolphthalein as an indicator. The resulting solution was extracted with dichloromethane (2x50ml), dried and the solvent removed *in vacuo* to yield a white solid, ethoxycarbonylmethylene-triphenyl phosphorane, 0.647g (2.04mmol, 95%).

Mp=115-119°C (lit. value[56] = 117°C). This ylide can be

stored at 0°C indefinately

δ_H ($CDCl_3$, 60MHz) 0.84 (3H, t, J=8Hz, $C\underline{H}_3$), 2.99 (2H, bs, $C\underline{H}_2$), 3.74 (2H, q, J=8Hz, $OC\underline{H}_2$), 6.75-7.5 (15H, m, aromatic protons).

To the aldehyde (173), (0.52g, 2.8mmol) in dry dichloromethane (50ml) was added the ylide (3.5g, 11.1mmol, 4equiv.) and the solution refluxed for 4 days under a N_2 atmosphere. After cooling, water (50ml) was added and the aqueous layer separated. The aqueous layer was re-extracted with diethyl ether (50ml). The organic layers were combined, dried and evaporated. The residue was cooled in an ice bath and the triphenyl phosphine oxide allowed to crystallize out. A small portion of diethyl ether was added and the precipitated triphenyl phosphine oxide filtered off. After evaporation of the solvent, the residue was further purified by flash column chromatography using hexane:ethyl acetate 1/4 as the eluting solvent. This yielded the THP ether of ethyl 3-hydroxy-2-methylhex-2-enoate (174), (0.69g, 2.7mmol, 96%).

δ_H ($CDCl_3$, 300.133MHz) *Inter alia*, 1.1-1.3 (9H, m, 3x$C\underline{H}_3$), 4.1-4.25 (2H, m, $OC\underline{H}_2$), 5.8, 5.85 and 5.9 (1H, 3xm, H-2).

The protected unsaturated ester (174), (0.69g, 2.7mmol), was hydrolysed to the corresponding sodium salt (175) by reaction with 2M NaOH (1.7ml, 1.5equiv.) in methanol (10ml). The aqueous solution was stirred at room temperature for 20h. The methanol was evaporated and water (20ml) was

added. This was extracted with hexane (3x20ml). The hexane extracts were combined and back extracted with water. The aqueous extracts were combined and the pH adjusted to 8 with 1M HCl solution. The water was removed as an ethanol azeotrope, to yield as a white solid, the sodium salt of the THP ether of 5-hydroxy-4-methylhex-2-enoic acid (175) which was used directly in the next reaction. The white solid (175), was reacted with triethylamine (0.38ml, 0.27g, 2.7mmol) and chloro diphenyl phosphinite (0.56ml, 0.73g, 2.7mmol) in dry dichloromethane, at -78°C under N_2 atmosphere, for 15min. After warming to room temperature the solution was stirred for a further 10min and then was diluted with diethyl ether and the amine hydrochloride by-product removed by filtration. The filtrate was washed with bicarbonate solution, dried and evaporated to produce an oil, the mixed phosphate anhydride (177). Again this was used directly in the next reaction.

SYNTHESIS OF THALLIUM (I) SALT OF N-(-2-MERCAPTOETHYL)ACETAMIDE.

This was prepared as described by Schwab and Klassen. To a dry, pre-weighed 250ml round bottomed flask equipped with a magnetic stirring bar and a rubber septum cap was added 3.72g (14.9mmol) of Thallium ethoxide. Following the addition to the flask of 145ml of nitrogen saturated THF, 1.86g (15.65mmol) of freshly prepared N-acetylcysteamine was added dropwise, via syringe, over a period of 20min. The concentration of Tl-NAC in the resulting bright yellow suspension (which was stored at 4°c) was calculated to be

0.098M.

The mixed phosphate anhydride (177), generated above, was taken up in dry degassed THF (70ml), under a N_2 atmosphere and cooled to 0^oC and Thallium (I) N-acetylcysteamine (27.5ml, 2.7mmol)was added to this solution. The resulting yellow suspension was stirred at 0^oC for 4h and then at room temperature overnight. The solution had changed colour from yellow to white during this time. The reaction mixture was concentrated *in vacuo* and the residue taken up in diethyl ether and filtered through celite. The filtrate was concentrated to yield the **THP ether of NAC thioester of racemic 5-hydroxy-4-methylhex-2-enoic acid (178).** The THP ether protection on (178) was removed by stirring in methanol (5ml) with 1 drop of trifluoroacetic acid, at room temperature overnight. The volatile materials were evaporated off and the residue taken up in ethyl acetate and washed with 5% $NaHCO_3$ solution and then dried and evaporated to dryness to yield the crude product (179), (0.25g, 1.02mmol, 38%, based on the amount of starting unsaturated ester). This was purified by preparative tlc using hexane:ethyl acetate 1/5 and 1 drop of triethylamine as the eluting solvent. This yielded 110mg of pure **NAC thioester of 5-hydroxy-4-methylhex-2-enoic acid (179).**

δ_H ($CDCl_3$, 300.133MHz) 1.1 (3H, d J=6Hz, $C\underline{H}_3$), 1.2 (3H, d J=6Hz, $C\underline{H}_3$), 1.9 (3H, s, $C\underline{H}_3CO$), 2.3-2.4 (1H, m, H-3), 3.05-3.1 (2H, m, $SC\underline{H}_2$), 3.4-3.45 (2H, m, $C\underline{H}_2N$), 3.7-3.8 (1H, m, H-4), 6.1-6.2 (1H, dd $J_{vicinal}$=15Hz, $J_{allylic}$=1Hz, H-2),

6.6 (br s, exchangeable NH), 6.9-7.0 (1H, dd J=15Hz, 7.5Hz, H-3).

Mass spectrum m/z 246 (M^+)
Calcd for $C_{11}H_{20}NO_3S$ (MH^+) 246.11638 ; found 246.1164000.

INCORPORATION STUDIES USING CHAIN-ELONGATION INTERMEDIATES

In all these studies, the modified culture and fermentation conditions were used.

INCORPORATION OF N-ACETYLCYSTEAMINE THIOESTER OF (2S,3S)-[2'-$^2H_3$]-3HYDROXYBUTANOIC ACID

In this incorporation experiment, ten second stage flasks were set up, as outlined previously. After 20h fermentation, the labelled precursor (500mg, as a solution in 3ml DMSO, *ie.* 0.3ml/flask) was added to the growing culture. The flasks were harvested, as detailed previously, after 40h fermentation and yielded methyl pseudomonate as a white solid (73mg).

INCORPORATION OF ETHYL (2S,3S)-[2'-$^2H_3$]-3-HYDROXYBUTYRATE

Again, ten second stage flasks were used in this feeding study. These were set up as detailed previously, and the labelled precursor was again added to the growing second stage culture after 20h, (500mg, as a solution in sterile water (1ml) *ie.*
0.1ml/flask). After 40h fermentation, the flasks were harvested to yield methyl pseudomonate as a white solid, (61.3mg).

INCORPORATION OF SODIUM (2S,3S)-[2'-$^2H_3$]-3-HYDROXYBUTYRATE

Again, ten second stage flasks were used in this feeding study. These were set up as detailed previously, and this labelled precursor added to the fermentation after 20h, (210mg as a solution in 10ml sterile water, *ie.* 1ml/flask). After 40 h fermentation the flasks were harvested and after workup yielded methyl pseudomonate as a white solid, (60mg).

INCORPORATION OF SODIUM [2-$^2H_3$]ACETATE

(1) Ten second stage flasks were set up as detailed previously and fed with [2-$^2H_3$]acetate after 20h growth, (250mg as a solution in sterile water 10ml, *ie.* 1ml/flask). These flasks were harvested after 40h fermentation. The pH of the culture supernatant was unusually low (pH 3.5). Extraction was carried out as described previously, except the pH of the supernatant was left unaltered. After workup, a colourless oil (20mg) was obtained which had a rf 0.3. This was shown by ^1H nmr to methyl pseudomonate.

(2) To take into account, the extra acidity of feeding [2-$^2H_3$]acetate, the second stage medium was buffered on the alkaline side, as detailed for the [1-^{13}C]acetate incorporation experiments. In this incorporation study, twenty second stage flasks were used and inoculated as described previously. After 20h fermentation [2-$^2H_3$]acetate (200mg as a solution in 10ml sterile water, *ie.* 0.5ml/flask) was added to the growing

culture. These flasks were harvested after 40h fermentation and the pH of the culture supernatant was normal (pH 8). After workup as described previously, this yielded methyl pseudomonate as a white solid (130mg).

GROWTH OF *P. fluorescens* IN A SECOND STAGE MEDIUM CONTAINING 5% ^2H_2O

The second stage culture medium containing all the constituents detailed previously was dissolved in ^2H_2O (50ml) and sterile water (950ml). This solution was dispensed into ten second stage flasks and autoclaved as detailed previously. These flasks were inoculated and grown up using the fermentation conditions outlined previously. They were harvested after 40h fermentation and yielded methyl pseudomonate as a white solid, (110mg).

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LECTURE COURSES

Postgraduate Lecture Courses

(1) Organic research seminars (2 years attendance at Edinburgh, and 1 year attendance at Leicester, Universities).

(2) Perspectives in Cell Biology by Dr J Phillips (Department of Biochemistry, Edinburgh University), (5 lectures).

(3) Medicinal Chemistry by Prof P G Sammes, (5 lectures).

(4) Natural Products (4th year lecture course) by Dr T J Simpson, (5 lectures).

(5) Molecular Probes by Prof P G Sammes, (5 lectures).

(6) Modern Methods of NMR by Dr I Sadler, (5 lectures).

(7) Recent Advances in Organic Chemistry by Prof R Ramage *et al*, (5 lectures).

(8) Business Management by Prof S Coke *et al*, (4 lectures).

(9) Department Technical German lectures and examination (1986-87).

PUBLICATION

Biosynthetic Studies on Pseudomonic Acid (Mupirocin), a Novel Antibiotic Metabolite of *Pseudomonas fluorescens*

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^{2}H and ^{18}O Isotope shifts observed in the ^{13}C n.m.r. spectra of pseudomonic acid (mupirocin) enriched from [1-^{13}C, $^{2}H_3$]- and [1-^{13}C, $^{18}O_2$]-acetates provide information on the mechanisms of formation of the tetrahydropyran and ester functions. The results of incorporation studies with ^{14}C- and $^{13}C_2$-labelled β-hydroxy-β-methylglutarates do not support previous proposals for its involvement in the biosynthesis of pseudomonic acid.

Pseudomonic acid A is a structurally unique antibiotic produced by *Pseudomonas fluorescens*[1] and which is now being used clinically under the generic name mupirocin for topical treatment of skin infections. It has a complex structure (1) consisting of a C_{17} unsaturated carboxylic acid entity containing epoxide, diol, and tetrahydropyran functions, esterified by a 9-hydroxynonanoic acid entity.[2] On the basis of preliminary biosynthetic studies[3] the pathway summarised in Scheme 1 was proposed. According to this, pseudomonic acid is essentially polyketide in origin and is elaborated *via* C_{12}, C_5, and C_9 units. Among the unusual features of the pathway are

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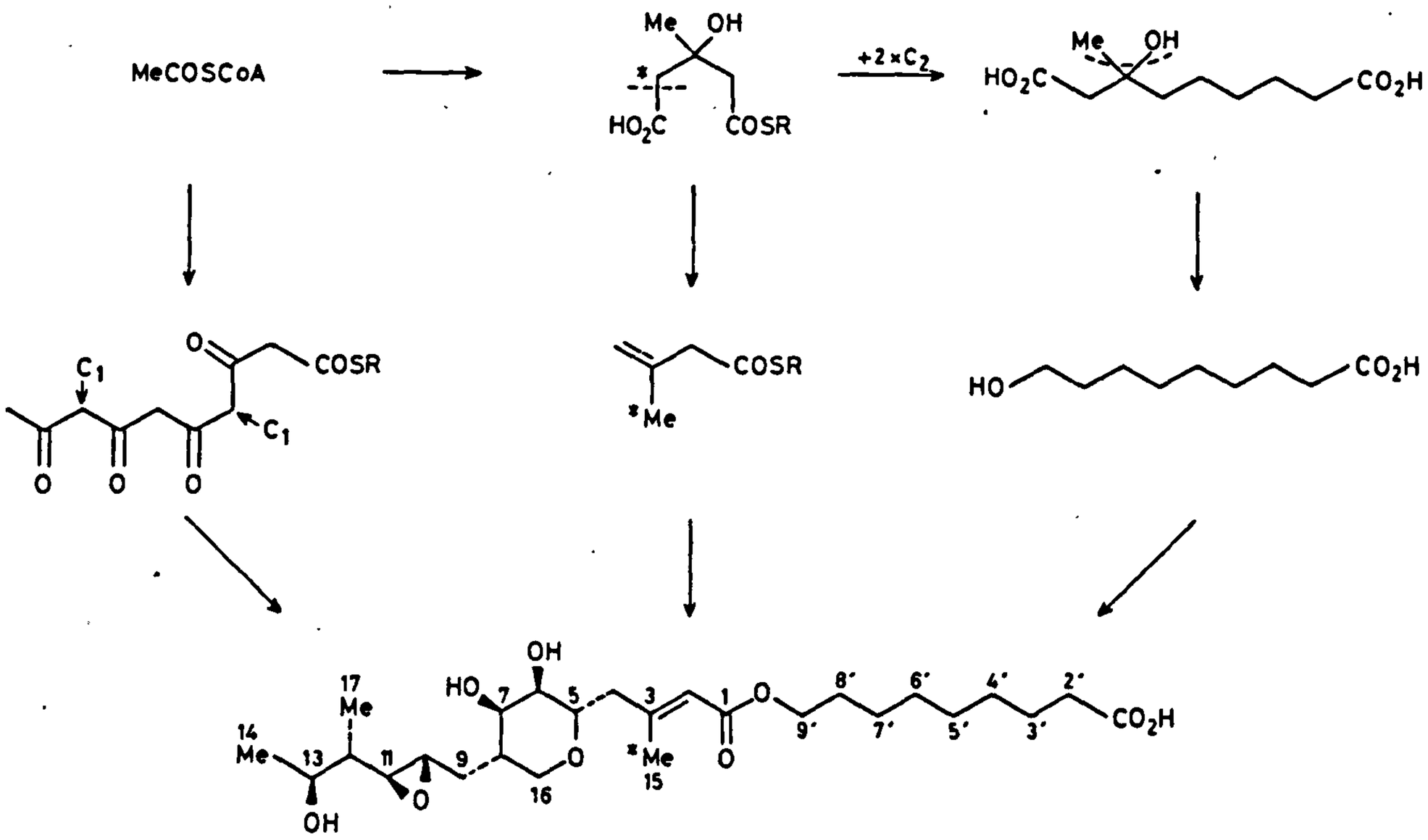
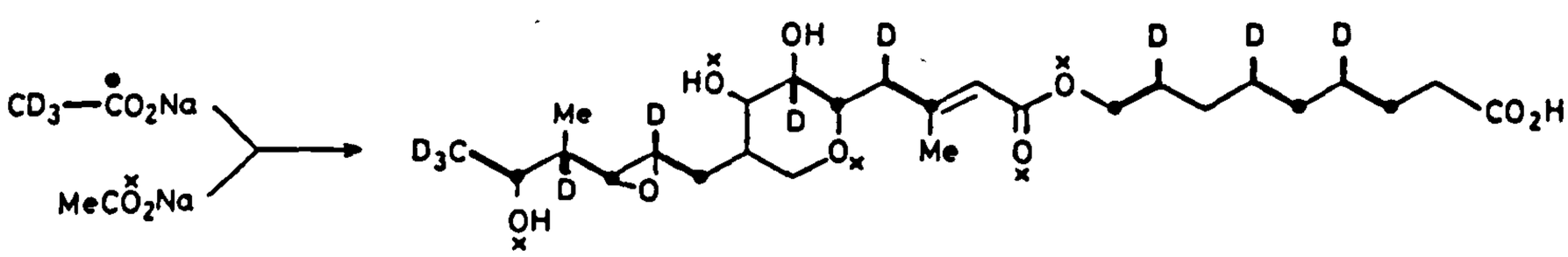
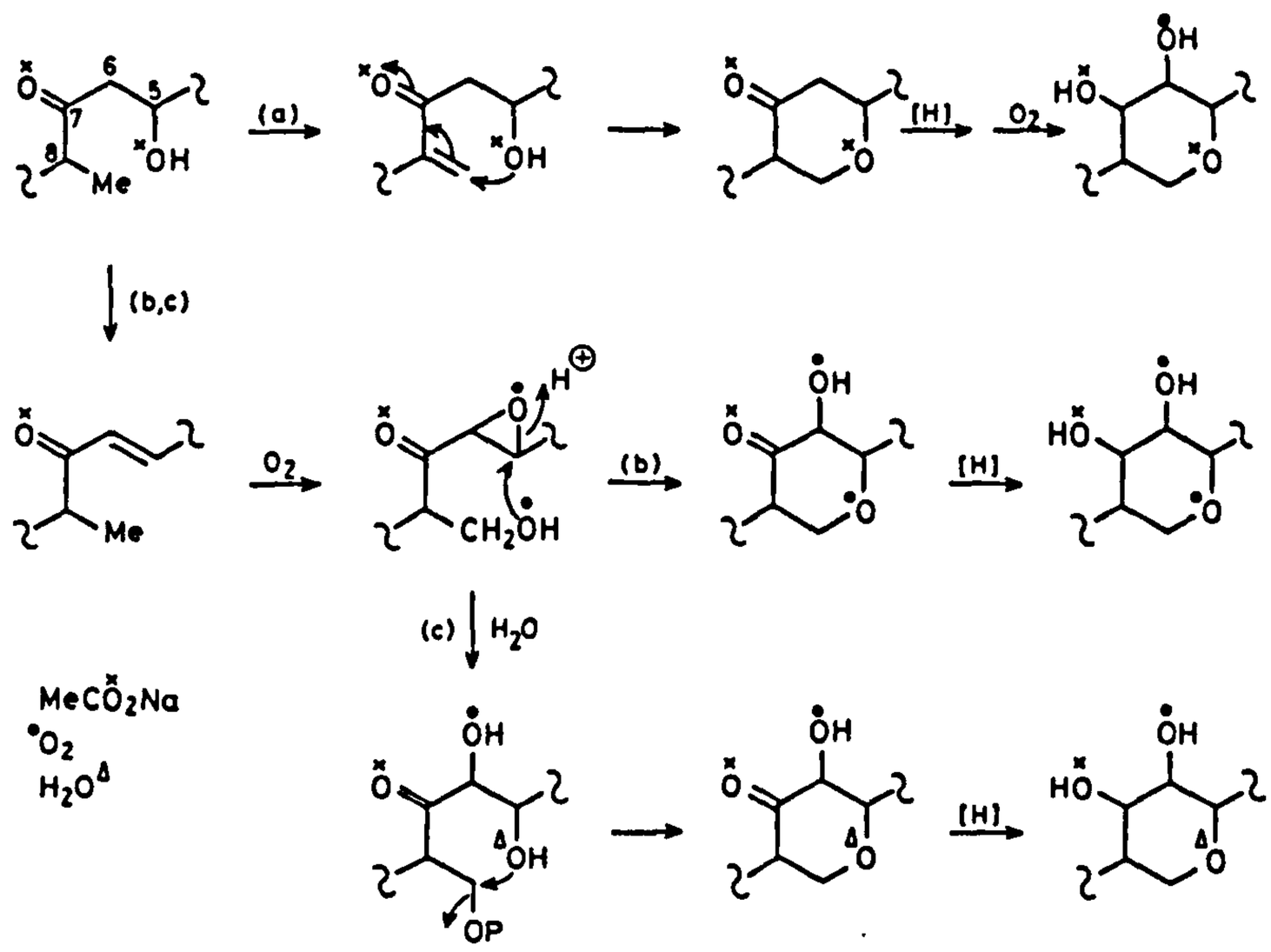


(1)

Scheme 1.



Scheme 2.



Scheme 3.

Table. ^{2}H and ^{18}O Isotope-induced shifts observed in the 90.56 MHz ^{13}C n.m.r. spectrum of pseudomonic acid A (1)

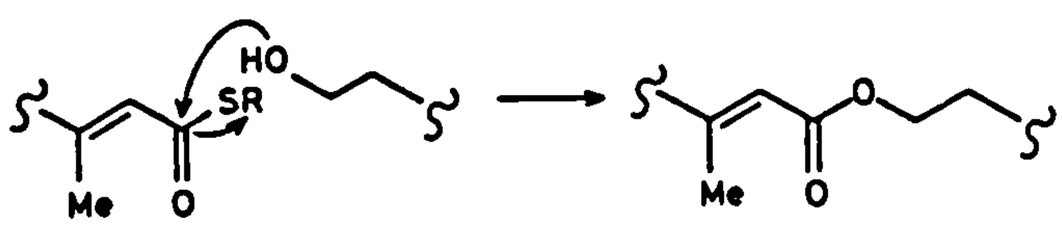
Carbon	δ_C (p.p.m.)	$\Delta\delta \times 100$ (p.p.m.)[a]	$\Delta\delta \times 100$ (p.p.m.)[b]
1	166.7	3.7	
3	156.5		5.1
5	74.7	1.7	5.5
13	71.3	2.3	4.9, 9.1, 13.7
7	70.3	2.3	
9′	63.7	2.9	2.9
11	61.2		7.2
9	31.5		13.7
5′	29.0		10.1
3′	24.8		9.8

[a] [1-^{13}C, $^{18}O_2$]Acetate-enriched. [b] [1-^{13}C, $^{2}H_3$]Acetate-enriched.

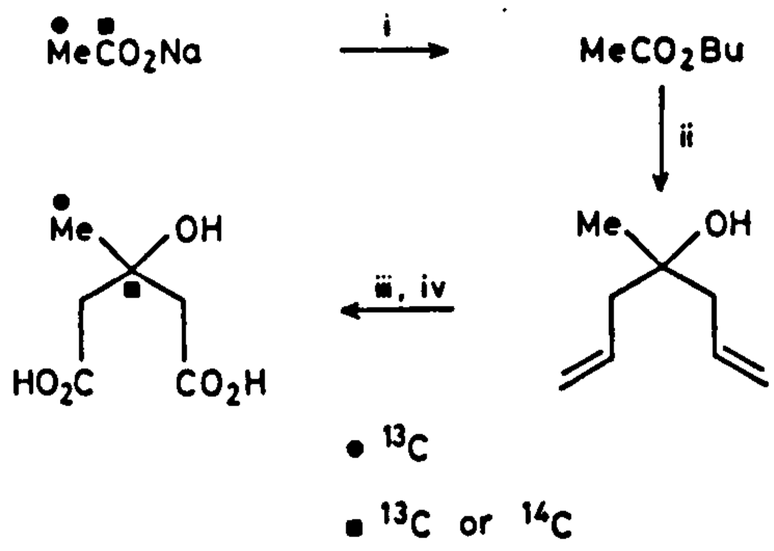
the proposed involvement of β-hydroxy-β-methylglutarate in the formation of both the C_5 and C_9 moieties and the origin of the C-15 methyl from the methyl carbon of a cleaved acetate unit. This feature has been observed more recently in the biosynthesis of virginiamycin,[4] myxovirescin,[5] and myxopyronin[6] but no satisfactory biosynthetic explanation has been established as yet.

A number of incorporation experiments with precursors labelled with stable isotopes which provide more information on the biosynthetic pathway to pseudomonic acid are now reported.

The ^{13}C n.m.r. spectrum of pseudomonic acid has been rigorously assigned.[7] Incorporations of [1-^{13}C, $^{18}O_2$]- and [1-^{13}C, $^{2}H_3$]-acetates and analysis of the enriched metabolites by highfield ^{13}C n.m.r. (Table) revealed the origins of the hydrogen, and more importantly, the oxygen atoms indicated in Scheme 2. The oxygens attached to C-1, C-5, C-7, C-13, and C-9′ are all derived from acetate. Of a number of biogenetically reasonable mechanisms which can be proposed for the formation of the tetrahydropyran moiety (Scheme 3) only path (a) is consistent with the oxygen-labelling results. These results also confirm that the ester linkage in pseudomonic acid is formed *via* separate C_{17} and C_9 moieties (Scheme 4) and not *via e.g.* a Baeyer-Villiger-type cleavage of a single long-chain ketone intermediate.



Scheme 4.



Scheme 5. *Reagents:* i, $(BuO)_3PO$, reflux; ii, CH_2=$CHCH_2Br$, Mg, tetrahydrofuran, Et_2O; iii, O_3, HOAc, CH_2Cl_2; iv, H_2O_2, HOAc

The proposed involvement of β-hydroxy-β-methylglutaric acid has been tested by the synthesis[8] of [3-^{14}C]- and [3,6-$^{13}C_2$]-β-hydroxy-β-methylglutarates (Scheme 5). The ^{14}C-labelled precursor was incorporated with high efficiency into pseudomonic acid (dilution value[9] 7.2). However, analysis of the ^{13}C n.m.r. spectrum of the metabolite derived from the $^{13}C_2$-labelled precursor, showed ^{13}C–^{13}C coupling satellites throughout the molecule, consistent with incorporation of label from the glutarate entirely *via* prior breakdown to acetyl coenzyme A and subsequent re-incorporation. There was no evidence whatsoever for the preferential enrichments of C-3 and C-4 or C-7′ required by the pathway shown in Scheme 1.

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